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The role of calcium during oocyte maturation, activation and ageing

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List of Abbreviations

2-aminoethoxydiphenyl borate (2-APB)
Acetoxymethyl (AM)
Anaphase-promoting complex/cyclosome (APC/C)
Area under the curve (AUC)
Assisted oocyte activation (AOA)
Assisted reproductive technologies (ART)
Bovine serum albumin (BSA)
Calcium ions (Ca^{2+})
Ca^{2+}/Calmodulin dependent protein kinase II (CaMKII)
Ca^{2+} stores in ER ($[Ca^{2+}]_{ER}$)
Capacitative Ca^{2+} entry (CCE)
Concentration of Ca^{2+} ($[Ca^{2+}]$)
COOK Cleavage medium (CC)
Cyclic adenosine monophosphate (cAMP)
Cyclin-Dependent Kinase 1 (CDK1)
Cytochalasin D (CCD)
Diacylglycerol (DAG)
Dimethyl Sulphoxide (DMSO)
Earle's Balanced Salt Solution (EBSS)
Endoplasmic reticulum (ER)
Energy Dispersive X-Ray Fluorescence spectrometer (EDXRF)
Germinal vesicle (GV)
Germinal vesicle breakdown (GVBD)
Human chorionic gonadotrophin (hCG)
Inositol 1,4,5-trisphosphate (IP_3)
Inositol 1,4,5-trisphosphate receptors (IP_3Rs)
Intracytoplasmic sperm injection (ICSI)
In vitro fertilization (IVF)
In vitro matured (IVM)
In vivo matured (IVO)
Metaphase of the first meiotic division (MI)
Metaphase of the second meiotic division (MII)
Metaphase promoting factor (MPF)
Minimum Essential Medium Alpha (α-MEM)
Mitogen-activated protein kinase (MAPK)
Phospholipase C (PLC)
Phospholipase C ζ (PLCζ),

Phosphatidylinositol 4,5-bisphosphate (PIP_2)

Plasma membrane Ca^{2+}-ATPase (PMCA)

Polar body (PB)

Post-acrosomal sheath WW domain-binding protein (PAWP)

Potassium simplex optimized medium (KSOM)

Pregnant mare serum gonadotrophin (PMSG)

Pronuclear (PN)

Protein kinase peA (PKA)

Quinns Advantage® Fertilization medium (SAGE)

Receptor operated Ca^{2+} channels (ROCs)

Relative amplitude (RA)

Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Ryanodine receptors (RYRs)

Sarcoplasmic reticulum (SR)

Sarco-endoplasmic reticulum Ca^{2+} ATPases (SERCA)

Spindle assembly checkpoint (SAC)

Store-operated Ca^{2+} entry (SOCE)

Strontium (Sr^{2+})

Transient receptor potential (TRP)

TRP cation channel, subfamily V, member 3 (vanilloid 3) (TRPV3)

Type 1 inositol 1, 4, 5-triphosphate receptor (IP_3R1)

Visible aggregates of tubular smooth ER clusters (SERs)

Voltage-operated channels (VOCs)

Zona pellucida (ZP)

Summary

Assisted Reproductive Technologies (ART) have rapidly evolved over the past decades. However, some forms of infertility remain difficult to overcome, with certain female-related factors particularly challenging to address. Overall, 0.1-1% of women exhibit severe oocyte maturation resistance in which pregnancy can rarely be achieved, while 1-5% of patients experience complete fertilization failure following intracytoplasmic sperm injection (ICSI). Furthermore, oocyte ageing, due to advanced female age (reproductive oocyte ageing) and prolonged *in vitro* culture (post-ovulatory ageing) is associated with decreased fertilization capacity and poor embryo quality – ultimately impairing ART treatment outcomes. At present, patients with recurrent maturation resistance and a lack of mature oocytes can only resort to oocyte donation, while assisted oocyte activation (AOA) may be applied for patients with fertilization failure. The treatment for reproductive oocyte ageing is also very limited, with antioxidant administration currently the only available treatment. Investigating the underlying mechanisms of oocyte maturation resistance, AOA and oocyte ageing is of vast importance and may shed light on the physiology of these processes in human, ultimately facilitating more effective diagnostic and therapeutic approaches.

Successful oocyte maturation and activation are mediated by inositol 1,4,5-trisphosphate receptors (IP_3Rs). These generate characteristic Ca^{2+}-oscillations in the oocyte, which are either released spontaneously or triggered by the sperm factor phospholipase C zeta. Disrupted Ca^{2+} signaling is linked to reduced fertilization rates and impaired embryo developmental potential. In mouse, altered Ca^{2+} signaling is reported in maturation resistant oocytes, failed to fertilized oocytes, as well as *in vitro* aged oocytes. This PhD research is focused on Ca^{2+}-signaling deficiencies, which affect these three processes in both human and mouse oocytes.

We primarily investigated the nuclear normality of human oocytes resistant to meiotic maturation and further examined treatment outcomes from a patient group exhibiting maturation resistance. In addition, we employ a mouse model to further study meiotic maturation resistance, allowing for a comprehensive analysis of Ca^{2+}-signaling related mechanisms. Furthermore, we define external factors present in culture media that influence ionomycin triggered AOA. Additionally, we investigate the efficiency of using strontium (Sr^{2+}) as a potential regulator of the Ca^{2+}-signaling pathway and a clinical AOA agent. Finally, we consider the difference of Ca^{2+} oscilation patterns in oocytes resulting from post-ovulatory and reproductive ageing.

Firstly, we defined patients presenting with an abnormally high number of immature oocytes at retrieval (>40%). These patients were more likely to exhibit severe maturation resistance. We

recruited these patients as the Study group, while patients presenting with a normal number of mature oocytes at retrieval (≥ 60%), were termed the Control group. Donated immature oocytes from both groups were collected. Following *in vitro* culture, oocytes were classified as maturation resistant and *in vitro* matured (IVM). Treatment outcomes were evaluated in both the Study and Control groups based on the presence of maturation resistant oocytes. Overall, similarly high aberrant spindle-chromosome complex configurations were observed in maturation resistant oocytes from both Study and Control groups. IVM oocytes from the Study group revealed a significantly higher percentage of misaligned chromosomes compared to Controls. Remarkably, Study patients with at least one maturation resistant oocyte, showed significantly reduced cumulative pregnancy and live birth rates compared to Control maturation resistant patients. To further investigate this etiology, we applied a mouse model for maturation resistance and revealed defective Ca^{2+}-signaling of maturation resistant oocytes at germinal vesicular breakdown (GVBD) and during parthenogenetic activation. In conclusion, appropriate treatment strategies, including clinical utilization of IVM oocytes from the Study group patients, warrant further investigation.

Secondly, we investigated the efficiency of two chemical AOA agents, ionomycin and Sr^{2+} to induce Ca^{2+} rises in both human and mouse oocytes. The effect of ionomycin and external Ca^{2+} concentrations on the Ca^{2+}-oscillatory pattern of both mouse and human oocytes was investigated, as well as the developmental potential of embryos generated from these oocytes. In mouse, we demonstrated that the application of 5µM ionomycin with KSOM or 10µM ionomycin in Ca^{2+}-free KSOM, significantly reduced the amount of Ca^{2+} flux and resulted in blastocyst formation failure compared to 10µM ionomycin with KSOM. This highlights the importance of optimal culture conditions during the oocyte activation process. Moreover, the Ca^{2+} rise in human oocytes provoked by 10µM ionomycin increased together with the total calcium concentration present in the commercial embryo culture medium used during AOA. More specifically, we observed reduced mouse embryonic developmental efficiency when performing AOA during 10 minutes in SAGE and Vitrolife medium in contrast to COOK medium, while embryo culture was performed in identical conditions (KSOM-Cook Blastocyst).

Furthermore, we demonstrated that despite the presence of its potential activating channel (TRPV3, transient receptor potential cation channel V3), Sr^{2+} failed to mediate Ca^{2+} rises in human oocytes. Both agonists of TRPV3, 2-aminoethoxydiphenyl borate (2-APB) and carvacrol promoted a single Ca^{2+} transient and activated more than half of the human oocytes exposed to these agents. However, Sr^{2+} was not capable of inducing Ca^{2+} rises and activation in human oocytes. The pattern of localization of the TRPV3 protein in human oocytes differed from the reported pattern in mouse oocytes. Therefore, the clinical application of Sr^{2+} to overcome fertilization failure after ICSI is questionable.

Finally, both Ca^{2+} oscillation patterns and embryo developmental potential, were investigated following the exposure of reproductive-aged, post-ovulatory aged IVM and *in vivo* matured (IVO) mouse oocytes, to Sr^{2+}. We found variable disruptions to Ca^{2+} oscillation patterns in post-ovulatory aged IVO oocytes and reproductive-aged IVM and IVO oocytes, during activation. Moreover, we revealed that the altered Ca^{2+} oscillation pattern of post-ovulatory aged IVO oocytes, further impaired embryo development. However, this was not the case in reproductive-aged IVM and IVO mouse oocytes. Furthermore, by extending the *in vitro* culture time to 20-24 hours post GVBD, Ca^{2+} oscillatory ability and further blastocyst development were restored in IVM mouse oocytes with impaired Ca^{2+} oscillatory ability.

Overall, these findings support the involvement of altered Ca^{2+} signaling in maturation resistant oocytes, oocytes undergoing AOA treatment, post-ovulatory and reproductive-aged oocytes, and shed light on the possible diagnostic approaches for female-related infertility.

Samenvatting

Geassisteerde reproductieve technologieën (Assisted Reproductive Technologies, ART) zijn de laatste jaren snel geëvolueerd. Toch blijven bepaalde vormen van, meestal vrouwelijk-geassocieerde, infertiliteit moeilijk te behandelen. Van alle vrouwen vertoont 0.1-1% ernstige eicel maturatie resistentie, waardoor er zelden een zwangerschap kan bekomen worden, terwijl er bij 1-5% een compleet bevruchtingsfalen na intracytoplasmatische sperma injectie (ICSI) plaatsvindt. Daarnaast zijn eicel veroudering door een oudere leeftijd van de dame in kwestie (reproductieve eicel veroudering) alsook door langdurige *in vitro* cultuur (post-ovulatoire eicel veroudering) bijkomende factoren die geassocieerd worden met een verlaagde bevruchtings capaciteit en verminderde embryo kwaliteit. Tegenwoordig kunnen patiënten met een eicelmaturatie resistentie en een tekort aan mature functionele eicellen enkel een beroep doen op eicel donatie. Geassisteerde eicel activatie (assisted oocyte activation, AOA) toegepast kan worden bij patiënten waarbij er gefaalde bevruchting plaatsvindt. De behandeling van reproductieve veroudering is daarnaast ook erg gelimiteerd, waarbij men tegenwoordig enkel de inname van antioxidanten kan adviseren. Het onderzoek naar de onderliggende mechanismen van eicel maturatie resistentie, AOA en eicel veroudering is van groot belang, om meer inzicht te verkrijgen in deze processen, en er uiteindelijk tot een meer effectieve diagnose en therapeutische behandeling kan gekomen worden.

Succesvolle eicel maturatie en activatie worden geregeld door inositol 1,4,5-trifosfaat receptoren (IP_3Rs). Deze receptoren genereren karakteristieke Ca^{2+} oscillaties in de eicel, die ofwel spontaan tot stand komen ofwel uitgelokt worden door de zaadcel factor fosfolipase C zeta. Abnormale Ca^{2+} signalisatie is verbonden met gereduceerde bevruchtingsratio's en een verlaagd embryo ontwikkelingspotentieel. In muis werd een gewijzigde Ca^{2+} signalisatie gerapporteerd in maturatie resistente eicellen, eicellen met gefaalde fertilisatie en *in vitro* verouderde eicellen. Dit doctoraatsonderzoek focust op Ca^{2+} signalisatie defecten die deze drie boven vernoemde processen beïnvloeden in zowel humane als muizen eicellen.

Eerst onderzochten we de normaliteit van de kern in humane eicellen die resistent zijn aan meiotische maturatie en keken we naar de klinische uitkomstparameters van deze patiëntengroep. Daarnaast gebruikten we ook een muismodel om meiotische maturatie resistentie verder te bestuderen, hetgeen een duidelijke analyse van de Ca^{2+} signalisatie gerelateerde mechanismen toelaat. Voor de toepassing van AOA hebben we nagegaan welke externe factoren die aanwezig zijn in het cultuurmedium die ionomycine getriggerde AOA kunnen beïnvloeden. Hierbij onderzochten we ook de efficiëntie van strontium (Sr^{2+}) als potentiële regulator van de Ca^{2+} signalisatie pathway en als klinisch AOA agens. Finaal bekeken we de verschillen tussen Ca^{2+} signalisatie patronen in eicellen die post-ovulatoire en

reproductieve veroudering vertonen.

We startten met het definiëren van patiënten met een abnormaal hoog aantal immature eicellen bij pick-up (>40%), aangezien deze groep met een grotere waarschijnlijkheid ernstige eicel maturatie resistentie zal vertonen. Deze patiënten werden geïncludeerd in de studie groep, terwijl patiënten met een normaal aantal mature eicellen bij pick-up deel uitmaakten van de controle groep. Gedoneerde immature eicellen van zowel de studie als de controle groep werden gecollecteerd. Na *in vitro* cultuur werden deze eicellen ingedeeld in twee groepen, maturatie resistent of *in vitro* gematureerd (IVM). Klinische uitkomstparameters werden zowel in de studie als de controle groep geëvalueerd op basis van de aanwezigheid van maturatie resistente eicellen. Er werd een gelijkaardig hoog aantal spoelfiguur-chromosoom complex abnormaliteiten geobserveerd in zowel de studie als de controle groep. IVM eicellen van de studie groep vertoonden een significant hoger percentage aan fout gealigneerde chromosomen in vergelijking met de controles. Opmerkelijk was dat patiënten met minstens één maturatie resistente eicel een significant lagere ratio vertoonden in cumulatieve zwangerschappen en levende geboortes in vergelijking met controle patiënten. Om dit fenomeen verder te onderzoeken pasten we een muizenmodel voor maturatie resistentie toe, waarbij er een defectieve Ca^{2+} signalisatie bij maturatie resistente eicellen tijdens germinale vesikel breakdown (GVBD) en tijdens parthenogenetische activatie aan het licht kwam. We kunnen concluderen dat desbetreffende behandelingsstrategieën, inclusief het klinisch gebruik van IVM eicellen, verder onderzocht moeten worden.

Daarna onderzochten we de efficiëntie waarmee twee chemische AOA agentia, ionomycine en strontium (Sr^{2+}), Ca^{2+} vrijstelling kunnen induceren in humane en muizen eicellen. Het effect van ionomycine en externe Ca^{2+} concentraties op het Ca^{2+} vrijstellingspatroon van zowel muis als humane eicellen werd onderzocht, alsook het ontwikkelingspotentieel van de embryo's die vanuit deze eicellen ontwikkelden. In muis konden we aantonen dat de toepassing van 5µM ionomycine in KSOM medium of 10µM ionomycine in Ca^{2+}-vrij KSOM medium, de hoeveelheid Ca^{2+} vrijstelling significant reduceerde in vergelijking met 10µM ionomycin in KSOM, hetgeen resulteerde in een gefaalde blastocyst vorming. Deze vaststelling wijst op het belang van de optimale cultuurcondities tijdens het eicelactivatie proces. De Ca^{2+} stijging in humane eicellen veroorzaakt door 10µM ionomycine steeg samen met de totale Ca^{2+} concentratie in het commerciële embryo cultuur medium dat tijdens AOA gebruikt werd. We observeerden een gereduceerde efficiëntie in de embryo ontwikkeling in muis na AOA in SAGE en Vitrolife medium ten opzichte van COOK medium, hoewel de embryocultuur in dezelfde cultuur omstandigheden plaatsvond (KSOM/Cook Blastocyst)

Verder toonden we ook aan dat Sr^{2+} faalde om de Ca^{2+} vrijlating in humane eicellen te induceren, ondanks de aanwezigheid van zijn potentieel activerend proteïne, TRPV3

(transient receptor potential cation channel V3). Beide agonisten van TRPV3, 2-aminoethoxydifenyl boraat (2-APB) en carvacrol, promootten één enkele Ca^{2+} piek en activeerden meer dan de helft van de humane eicellen die blootgesteld werden aan deze agentia. Toch was Sr^{2+} niet in staat om Ca^{2+} vrijstelling en activatie te induceren in humane eicellen. Het patroon van localisatie van TRPV3 eiwit in humane eicellen verschilde van het gerapporteerde patroon in muizen eicellen. Daarom wordt de klinische toepassing van Sr^{2+} om bevruchtingsfalen na ICSI te behandelen in vraag gesteld.

Finaal werd zowel het Ca^{2+} oscillatiepatroon als het embryo ontwikkelingspotentieel onderzocht na blootstelling van reproductief verouderde, post-ovulatoire verouderde IVM en *in vivo* gematureerde (IVO) muizen eicellen aan Sr^{2+}. We vonden variabele onderbrekingen in het Ca^{2+} oscillatiepatroon na activatie van post-ovulatoire verouderde IVO eicellen en reproductief verouderde IVM en IVO eicellen. We vonden ook dat het veranderde Ca^{2+} oscillatiepatroon van post-ovulatoire verouderde IVO eicellen de embryo ontwikkeling verder negatief beïnvloedde. Dit werd niet geobserveerd in reproductief verouderde IVM en IVO muizen eicellen. Verder konden we door de *in vitro* cultuur uit te breiden tot 20-24h post GVBD, de Ca^{2+} vrijlating en blastocyst ontwikkeling herstellen in IVM muis eicellen met een verminderd Ca^{2+} vrijstellingspotentieel.

Samengevat ondersteunen deze bevindingen het belang van Ca^{2+} signalisatie in maturatie resistente eicellen, eicellen die AOA ondergaan, post-ovulatoire en reproductief verouderde eicellen, en brengen ze mogelijke diagnostische en therapeutische benaderingen voor vrouwelijk-gerelateerde infertiliteit aan het licht.

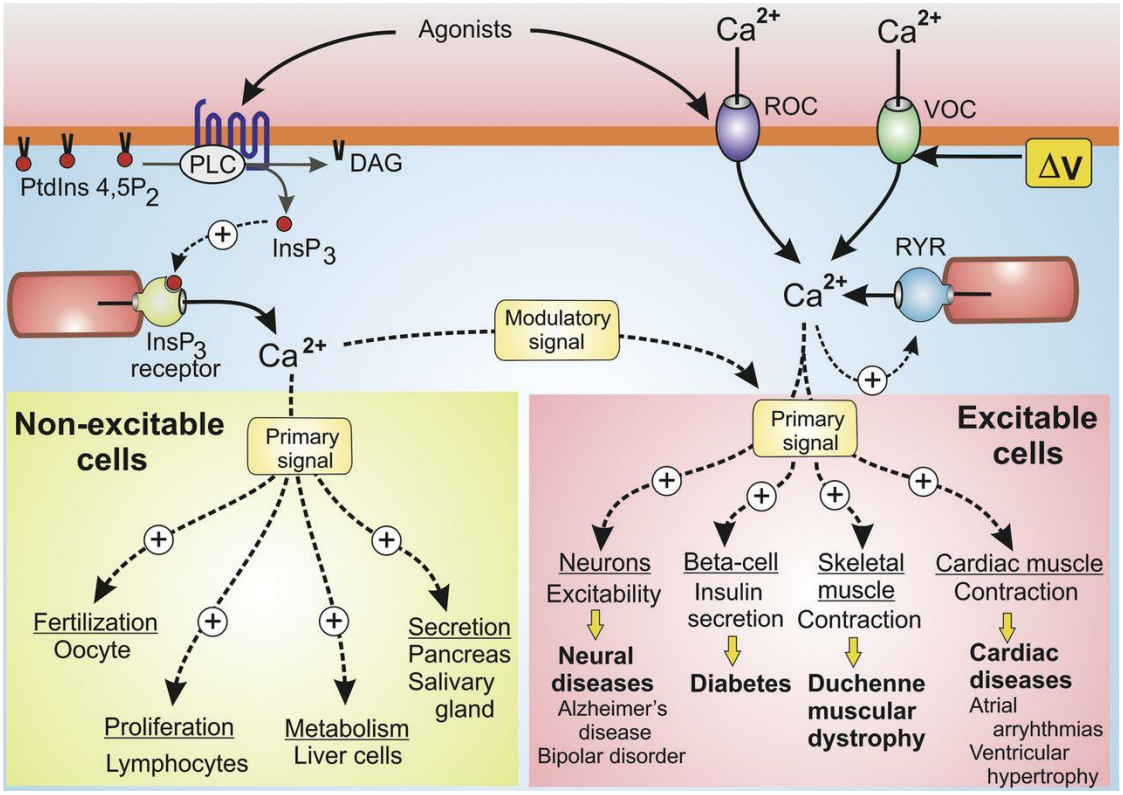
Chapter 1

1. General Introduction

1.1. The general physiological role of calcium

Calcium ion (Ca^{2+}) is known as a ubiquitous second messenger that regulates numerous cellular processes, ranging from secretion, contraction, metabolic enzymes, gene expression, to sensory signaling cascades and programmed cell death (Debant *et al.*, 2015). Cells exhibit an extremely low permeability to inorganic Ca^{2+}, due to the presence of a surrounding phospholid bilayer. To regulate cellular processes, an elevation in the concentration of intra-cytoplasmic Ca^{2+} by Ca^{2+} influx through the plasma membrane or Ca^{2+} release from intracellular Ca^{2+} stores, as well as the cross-talk between the Ca^{2+} and cyclic adenosine monophosphate (cAMP) signaling pathways are all required (Toescu and Dayanithi, 2012). Interestingly, different cytosolic Ca^{2+} signaling pathways are processed by excitable and non-excitable cells (Berridge, 2016) (Figure 1).

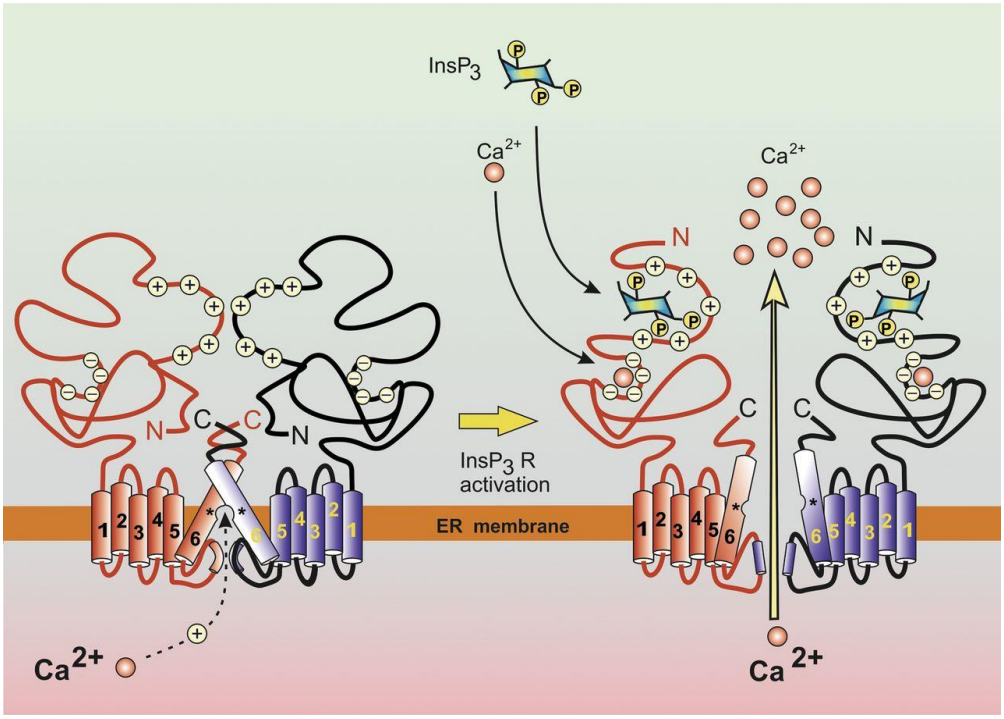
Figure 1. Distinct Ca^{2+} signaling pathways in excitable and non-excitable cells. Figure reprinted with permission. (Berridge, 2016)



In excitable cells, the primary Ca^{2+} signaling depends on the entry of Ca^{2+} through voltage-operated channels (VOCs). This is a class of transmembrane proteins, which is activated by alterations in the electrical membrane potential. This signaling pathway regulates processes such as memory formation in neurons, insulin secretion from beta cells and contraction in muscle cells (Berridge, 2016) (Figure 1). Moreover, receptor-operated Ca^{2+} channels (ROCs) also play a role. For instance, ROCs are responsible for the Ca^{2+} entry into smooth muscle cells, to sustain cellular activation (Gibson *et al.*, 1998) and ascribe the Ca^{2+} inflow in neurons, the classic example of an excitable cell (Moccia *et al.*, 2015). The function of ROCs is modulated crucially on the action of agonists, antagonists and particular compounds that regulate receptors. Recently, the emergence of the transient receptor potential (TRP) cation family underpins different ROCs pathways in excitable cells. Furthermore, plasma membrane store-operated Ca^{2+} entry (SOCE) channels are also involved in Ca^{2+} influx to refill intracellular Ca^{2+} stores of excitable cells. The activation of SOCE engages certain, specific Ca^{2+}-dependent processes, such as neuronal excitation (Moccia *et al.*, 2015). Besides the Ca^{2+} permeable channels, intracellular localized Ca^{2+} signaling from the endo- and sarcoplasmic reticulum (ER/SR) are also important for generating Ca^{2+} signals in excitable cells. In this regard, Ca^{2+} release by ryanodine receptors (RYRs) in internal stores enhances primary Ca^{2+} signaling generated by VOCs (Berridge, 2016).

Generally, non-excitable cells have only ROCs, and show a lack of VOCs. The primary Ca^{2+} signals in non-excitable cells are generated by inositol 1,4,5-trisphosphate (IP_3), control processes as diverse as metabolism, secretion, fertilization and proliferation (Berridge, 2009; Mikoshiba, 2015) (Figure 1). To form IP_3, external stimuli, for instance, neurotransmitters, hormones and growth factors activate either the G protein-coupled receptors or the protein tyrosine kinase-linked receptors that are coupled to different phospholipase C (PLC) isoforms (Cocco *et al.*, 2015). Further downstream, the activated PLC hydrolyzes the precursor lipid phosphatidylinositol 4,5-bisphosphate (PIP_2) to form both diacylglycerol (DAG) and IP_3. The IP_3 functions by further binding to the IP_3 receptors (IP_3Rs) to release Ca^{2+} from the ER into the cytoplasm, therefore, elevate the Ca^{2+} load (Fedorenko *et al.*, 2014; Taylor *et al.*, 2014; Mak and Foskett, 2015; Mikoshiba, 2015) (Figure 2). This IP_3/Ca^{2+} signaling pathway further plays a modulatory role in the excitable pathway to induce subtle changes in the generation and function of their primary Ca^{2+} signal (Berridge, 2016).

Figure 2. IP_3 binding to IP_3R. Figure reprinted with permission. (Berridge, 2016)



The characteristic feature of the IP_3/Ca^{2+} signaling pathway is that it normally generates Ca^{2+} release as oscillations. When sustained Ca^{2+} release is required for a longer period, repetitive increases occur as Ca^{2+} oscillations (Berridge, 1993; Fewtrell, 1993). The oscillations have been described in multiple cell types, from hepatocytes (Woods *et al.*, 1986; Rooney *et al.*, 1989), osteoclasts (Takayanagi *et al.*, 2002), astrocytes (De Pittà *et al.*, 2009), renal epithelial cells (Aizman *et al.*, 2001), mesoepithelial cells (Pecze and Schwaller, 2015), endothelial cells (Moccia *et al.*, 2003) to oocytes (Cuthbertson and Cobbold, n.d.; Cheek *et al.*, 1993).

The theory of oscillator was suggested initially as the periodic sensitization of the IP_3R, which is caused by the refilling of the intracellular store through the external Ca^{2+} entry (Berridge, 2007). Later, it has been complemented by the re-uptake of Ca^{2+} into the ER following each transient. More recent studies indicate that the oscillatory mechanism may either involve complex feedbacks regarding IP_3 and Ca^{2+} regulations, as the level of IP_3 oscillates together with the level of Ca^{2+} (Hajnóczky and Thomas, 1997; Politi *et al.*, 2006; Zhang *et al.*, 2011; Gaspers *et al.*, 2014) or is triggered by a constant concentration of IP_3, independent of the Ca^{2+} oscillation period (Sneyd *et al.*, 2017). Gene expression is initiated more effectively by Ca^{2+} oscillations than by a steady Ca^{2+} release (Li *et al.*, 1998). Moreover, the frequency of Ca^{2+} oscillations regulates the activity of kinases, for instance protein kinase CaM kinase II, and thereby contributes to multiple cellular processes, such as neuronal plasticity, cell excitability and secretion (De Koninck and Schulman, 1998).

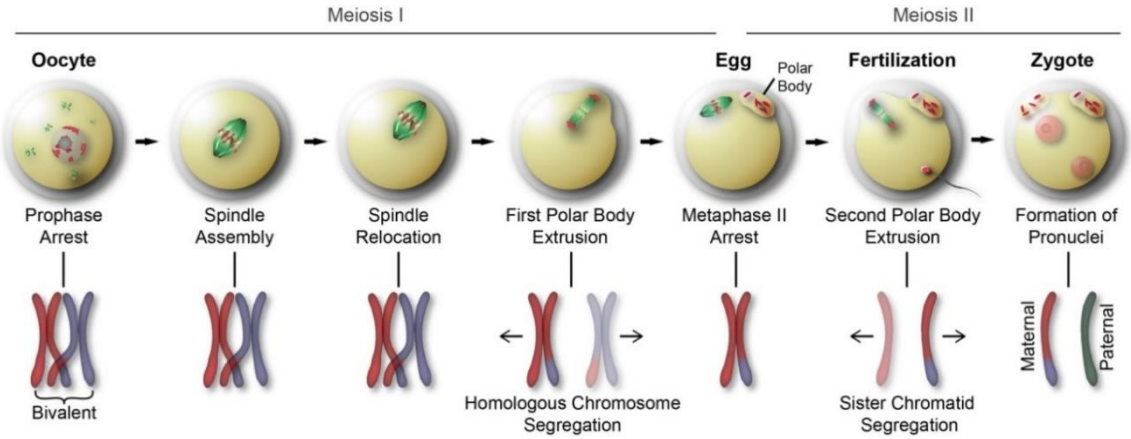
1.2. IP_3/Ca^{2+} signaling during oocyte maturation and early embryo formation

1.2.1 The story of oocyte maturation and oocyte activation

In mammals, oogenesis initiates at the embryonic stage when primordial germ cells commence meiosis and develop to primary oocytes (Baltus *et al.*, 2006). By birth, females have developed a finite number of primary oocytes arrested in meiotic prophase that comprise their lifespan egg supply (Lei and Spradling, 2013). At puberty, an increase of luteinizing hormone triggers the resumption of meiosis. It is marked by the breakdown of the large nuclear membrane of the oocyte, defined as germinal vesicle breakdown (GVBD).

The resumption and progression of meiosis refers to both nuclear and cytoplasmic meiotic maturation. To process nuclear maturation from metaphase of meiosis I (MI) to metaphase of the second meiotic division (MII) arrest (Eppig, 1996), the chromosomal DNA is replicated (MacLennan *et al.*, 2015). At the MI stage, homologous pairs move together along the metaphase plate by means of the meiotic spindle, and homologous chromosomes are pulled apart by shortening of the spindle in anaphase I (Page and Hawley, 2003; Baudat *et al.*, 2013). Once separated, the nuclear membrane reforms and the spindle structure depolymerizes in telophase I, and a 2n haploid number of chromosomes is left by extruding the first polar body (PB) containing half the genetic material, but very little of the cytoplasmic contents (Sharma *et al.*, 2013). Following PB extrusion, oocytes progress uninterruptedly to the MII stage and become arrested for a second time (Figure 3). Following both nuclear and cytoplasmic maturation, the oocyte is subsequently released from its associated somatic follicular compartment at the time of ovulation.

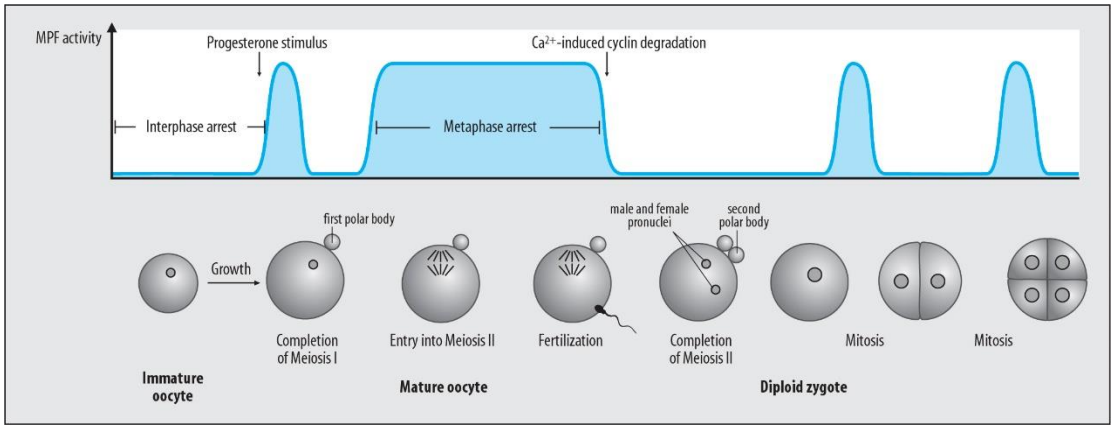
Figure 3. From oocyte meiosis maturation to early embryo formation. Figure reprinted with permission. (Clift and Schuh, 2013)



Cytoplasmic maturation occurs simultaneously with nuclear maturation, which consists of a number of changes necessary for acquisition of meiotic competence and subsequent oocyte activation and embryonic development (Sharma *et al.*, 2013). This entails the relocation of organelles and cytoskeleton, the accumulation of mRNAs, extensive protein synthesis and subsequent post-translational modifications (Epel, 1990; Carroll *et al.*, 1996).

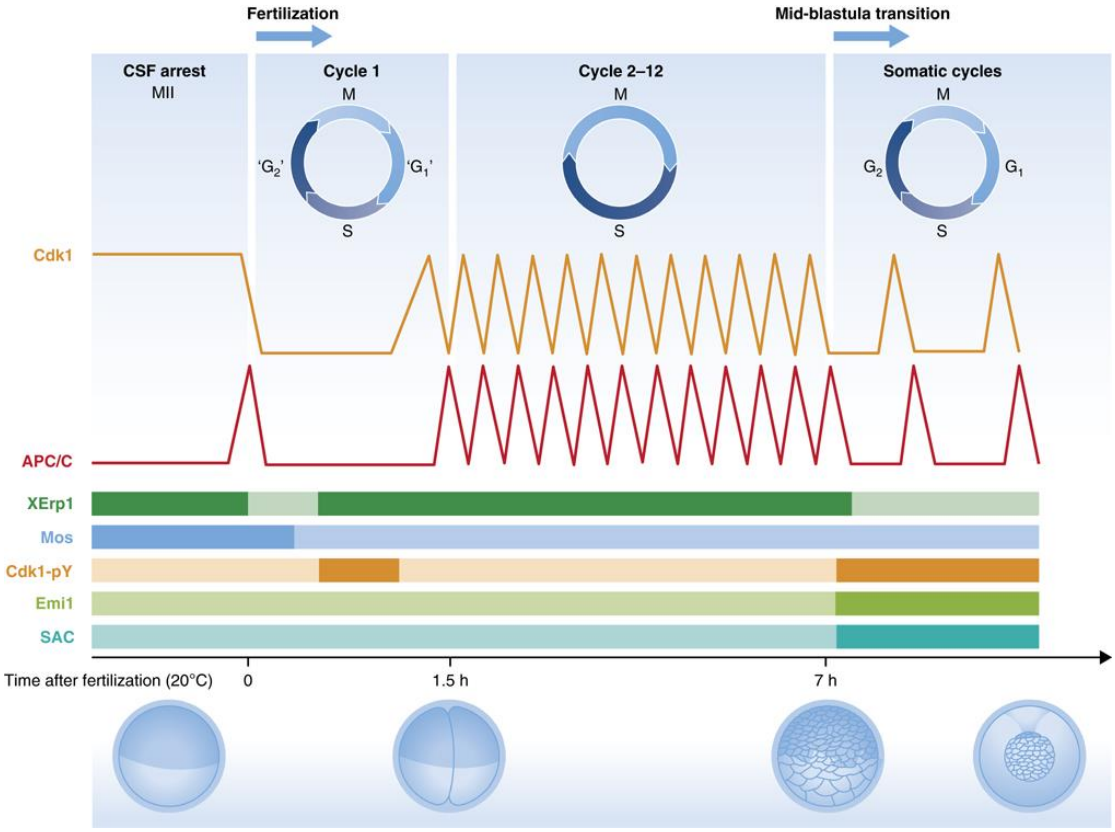
The protein changes regulate the resumption of meiosis. The re-initiation of meiosis coincides with a drop in intra-cytoplasmic cAMP concentration (Schultz *et al.*, 1983; Vivarelli *et al.*, 1983), which regulates the key protein complex Meta-phase promoting factor (MPF) that is formed from the Cyclin-Dependent Kinase 1 (CDK1) and kinase cyclin B (Jones, 2004) (Figure 4). A high concentration of intra-cytoplasmic cAMP activates protein kinase peA (PKA) (Viste *et al.*, 2005), which in turn, phosphorylates the phosphatase CDC25 and ensures the activity of CDK1 (Jones, 2004). The reduced cAMP level inhibits MPF activity, and as a direct consequence, meiotic maturation resumes (Jones, 2004).

Figure 4. Activity of MPF during oocyte meiotic maturation. Figure reprinted with permission. (Molecular & Developmental Biology (BIOL3530))



Beside MPF, the balance between oocyte arrest and progression also depends on alternating levels of another key mediator: anaphase-promoting complex/cyclosome (APC/C). Resumption of meiosis from the diplotene arrest is controlled by the activation of MPF (Jones, 2004). Exit out of prophase I and entry into M phase of meiosis I is hallmarked by the GVBD and the formation of the meiotic spindle. When the chromosomes are all properly aligned, the spindle assembly checkpoint (SAC) signal is extinguished, resulting in APC/C activation (Homer, 2013) (Figure 5). Thereafter, the oocyte progresses to the MII stage and remains arrested at this stage until it is fertilized.

Figure 5. APC/C and MPF activity during oocyte to embryo transients. Figure reprinted with permission. (Hörmanseder *et al.*, 2013)



Ca^{2+} signaling pathways are facilitated with these cytoplasmic changes. During cytoplasmic maturation, Ca^{2+} stored in the ER is elevated, while the number of Ca^{2+} channels, IP_3Rs, and their sensitivity to IP_3 are also increased (Murnane and DeFelice, 1993; Xu *et al.*, 2003).

During oocyte maturation, the primary Ca^{2+} store (ER) in the oocytes, dynamically changes during oocyte maturation. In GV oocytes, the ER accumulates in the inner cytoplasm. At GVBD, the ER forms a dense ring in the center of the oocyte around the MI spindle. In the later stage of maturation, the ER ring moves together with the MI spindle towards the oocyte cortex (FitzHarris *et al.*, 2007). During the dynamic relocation of the ER, the concentration of stored Ca^{2+} is elevated.

The number of type 1 IP_3 receptor (IP_3R1) increases during maturation, with MII oocytes containing almost two times more receptors than GV oocytes (Parrington *et al.*, 1998). The distribution of IP_3R1 also changes during oocyte maturation. In mouse GV oocytes, the IP_3R1 are spread throughout the cytoplasm forming a thin layer in the cortex (Fissore *et al.*, 1999). In human oocytes, the IP_3R1 form a patch-like structure in the cortex to facilitate the initiation of Ca^{2+} release or enhance their sensitivity (FitzHarris *et al.*, 2007). All these changes are crucial for establishing the ability to generate Ca^{2+} oscillations by the oocyte.

The initiation of the second meiotic chromosome segregation occurs following fertilization by sperm (Sharma *et al.*, 2013). This 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 the embryo. The oocyte has to be released from the MII 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). After fertilization, oocytes provides half of the nuclear genetic material, but endow the embryo with nearly all membrane and cytoplasmic determinants that are required for successful species propagation in sexually reproducing organisms (Baudat *et al.*, 2013). Over the next few days, the mammalian embryo undergoes a series of cell divisions, ultimately leading to the formation of a blastocyst (Aplin and Ruane, 2017).

1.2.2 Ca^{2+} homeostasis during oocyte maturation

At oocyte meiosis, Carroll *et al.* first reported that spontaneous Ca^{2+} fluctuations existed during *in vitro* maturation (IVM) of mouse oocytes. The intracellular Ca^{2+} is released through the IP_3R1 at GVBD and is crucial for successful cytoplasmatic maturation (Carroll and Swann, 1992). Fully grown mammalian GV oocytes are arrested and endowed with low Ca^{2+} reserves in ER ($[Ca^{2+}]_{ER}$). As maturation progresses, $[Ca^{2+}]_{ER}$ increases steadily until the MII stage (Tombes *et al.*, 1992; Jones *et al.*, 1995), while the most drastic increase in $[Ca^{2+}]_{ER}$ is observed around GVBD (Wakai *et al.*, 2013), which enhances IP_3R1-mediated Ca^{2+} release and promotes the acquisition of further fertilization-like oscillations (Mehlmann and Kline, 1994).

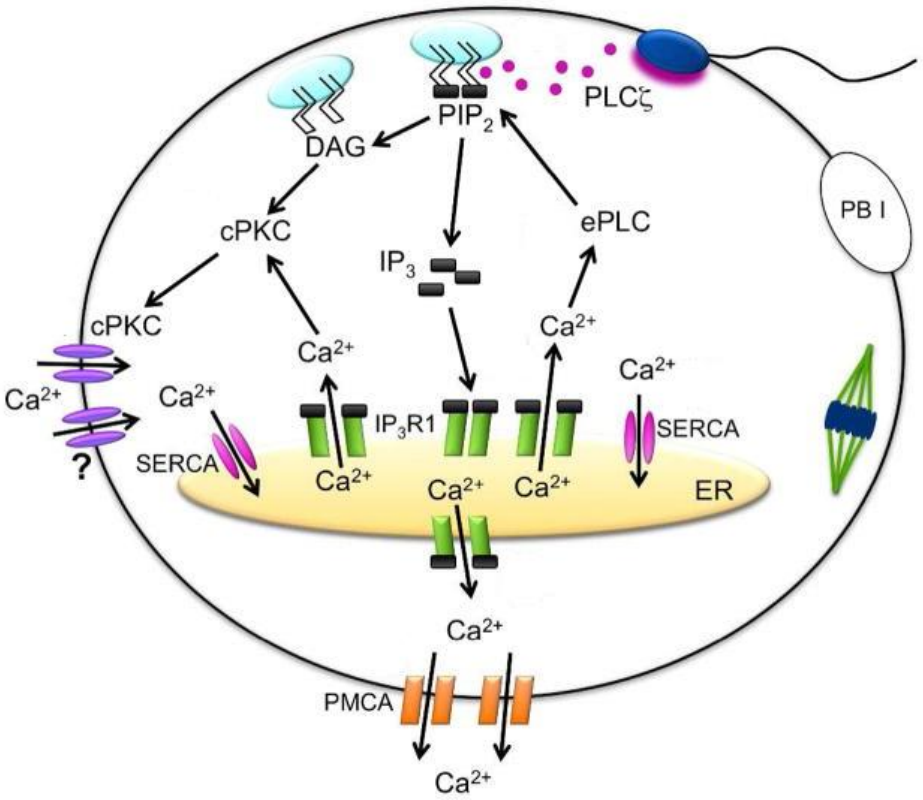
During maturation, the elevation of $[Ca^{2+}]_{ER}$ requires the function of both Ca^{2+} influx and efflux. The low levels of $[Ca^{2+}]_{ER}$ at the GV stage are attributed to the constitutive Ca^{2+} leak out of the ER, and thereafter promote Ca^{2+} influx to display the necessary intracellular Ca^{2+} oscillations. Ca^{2+} influx ceases around the time meiosis is resumed (Carroll and Swann, 1992) and the first increase in $[Ca^{2+}]_{ER}$ content occurs simultaneously (Jones *et al.*, 1995). The association between $[Ca^{2+}]_{ER}$ content and intracellular Ca^{2+} is related to the plasma membrane Ca^{2+}-ATPase (PMCA) and SOCE (Putney *et al.*, 1986; Hoth and Penner, 1993), as well as alterations of active Ca^{2+} influx during maturation (Cheon, *et al.*, 2013). The function of the Ca^{2+} influx via SOCE and efflux through PMCA pathways is significantly downregulated at the GVBD stage (Norwood *et al.*, 2000; El-Jouni *et al.*, 2005), which ensures the high concentration of $[Ca^{2+}]_{ER}$ around GVBD and supports the further sperm-associated intracellular Ca^{2+} responses in oocytes (Wakai and Fissore, 2013).

1.2.3 Generation of Ca^{2+}-signals during development: from fertilization to early embryo

Before the sperm can fertilize the oocyte, they must become hyperactived, to enable transport through the mucous layer within the oviduct and to penetrate the zona pellucida, to react with the oocyte plasma membrane. The process of hyperactivity is triggered by a Ca^{2+} increase in the redundant nuclear envelope that surrounds the sperm axoneme, which is mediated via IP_3Rs, as well (Ho and Suarez, 2001). Vitamin D which acts in a nongenomic pattern to activate the formation of the IP_3, is responsible for this Ca^{2+} release (Jensen, 2014).

At fertilization, the hyperactivated sperm fuses with the oocyte and releases its testis-specific phospholipase C ζ (PLCζ), which is unique to sperm. PLCζ hydrolyzes PIP_2 to form IP_3, which binds to its receptor IP_3Rs that localized on the ER, and thereby triggers the Ca^{2+} release (Swann and Lai, 2013; Kashir *et al.*, 2014) (Figure 6). Recent data have emerged, suggesting the sperm factor may be a post-acrosomal sheath WW domain-binding protein (PAWP) (Aarabi *et al.*, 2014). The importance of PAWP during the fertilization process seems low, as PAWP knockout mouse sperm is capable of triggering Ca^{2+} release and fertilization in mouse oocytes, while oocytes from PAWP knockout mice can also be fertilized (Nomikos *et al.*, 2014; Amdani *et al.*, 2015; Kashir *et al.*, 2015).

Figure 6. PLC-Z triggered IP_3/Ca^{2+} signaling pathway. Figure reprinted with permission. (Miao and Williams, 2012)



During fertilization, a single Ca^{2+} peak was initially described in marine animals as an explosion, suggesting this pattern may be responsible for oocyte activation (Ridgway *et al.*, 1977; Steinhardt *et al.*, 1977). Later, one single Ca^{2+} transient was demonstrated in oocytes from most non-mammals, during activation (Busa and Nuccitelli, 1985; Gillot and Whitaker, 1994), excluding eggs from nemertean worms, bivalve etc. (Stricker, 1999). Conversely, oscillations which sustain for several hours and terminate around the moment of pronucleus formation are observed in mammalian species (Jones *et al.*, 1995; Miao and Williams, 2012). The amplitude and frequency of the Ca^{2+} oscillation are crucial for the success of oocyte activation and further embryo development (Swann and Ozil, 1994; Miyazaki and Ito, 2006; Kashir *et al.*, 2014).

In both human and mouse oocytes, the initial surge of free cytoplasmic Ca^{2+} starts from the site of sperm penetration and expands as a wave through the oocyte (Swann, 1993). Subsequently, the intracellular cytoplasmic Ca^{2+} level starts to rise and persists for several minutes before returning to the baseline (Miao *et al.*, 2012). The amplitudes of these sperm triggered Ca^{2+} oscillations range from 0.5-2.5 µM, while the approximate duration of individual Ca^{2+} transient is about 0.5 to 4 minutes (Stricker, 1999). However, there is also species-specific differences. Following the injection of the same human sperm into both human and mouse oocytes, mouse oocytes show a 20 times higher frequency of Ca^{2+} peaks during the first 2 hours of measurement. Conversely, human oocytes exhibit 1-2 peaks/hour during the 10-16 hours of measurement after ICSI with human sperm. To compare the Ca^{2+} signaling generated in different species, the parameter that reflects the total amount of Ca^{2+} flux should be used.

During each oscillatory Ca^{2+} transient, there is a rapid decline in the $[Ca^{2+}]_{ER}$ and the level of $[Ca^{2+}]_{ER}$ is gradually replenished through SOCE. The buildup of $[Ca^{2+}]_{ER}$ sensitizes the IP_3Rs resulting in the activation of the next transient. These sperm-triggered Ca^{2+} oscillations induce chromosome separation and cell proliferation that occurs during early development. At mitosis, the local Ca^{2+} transients precede cell cleavage, which includes the lateral extension and deepening of the furrow (Gillot and Whitaker, 1994).

1.2.4 Regulation of Ca^{2+} oscillatory mechanisms during fertilization

Although oocytes only experience very small changes in membrane potential at fertilization (Jaffe *et al.*, 1983) and the primary Ca^{2+} oscillating mechanism has been described as the IP_3/Ca^{2+} signaling pathway, the regulation of the intracellular Ca^{2+} responses of mammalian oocytes is far more complex. To sustain Ca^{2+} oscillations without attenuation after each Ca^{2+} transient, Ca^{2+} levels have to be rapidly returned to baseline values, and the $[Ca^{2+}]_{ER}$ has to be refilled in anticipation of the next Ca^{2+} rise (Berridge, n.d.). To drive intracellular Ca^{2+} back to baseline levels, oocytes need to either take up the free cytosolic Ca^{2+} into the ER refilling the

store by the sarco-endoplasmic reticulum Ca^{2+} ATPases (SERCA) and/or remove it to other organelles or to the extracellular space surrounding environment by the action of PMCA (83) and Na^{+}/Ca^{2+} exchanger ("Na+-Ca2+ exchange in mouse oocytes: modifications in the regulation of intracellular free Ca2+ during oocyte maturation. - PubMed - NCBI," n.d.). Nevertheless, to consistently replenish $[Ca^{2+}]_{ER}$ and maintain long lasting oscillations, extracellular Ca^{2+} influx across the plasma membrane is required by a variety of channels, for instance, SOCE (Smyth *et al.*, 2006).

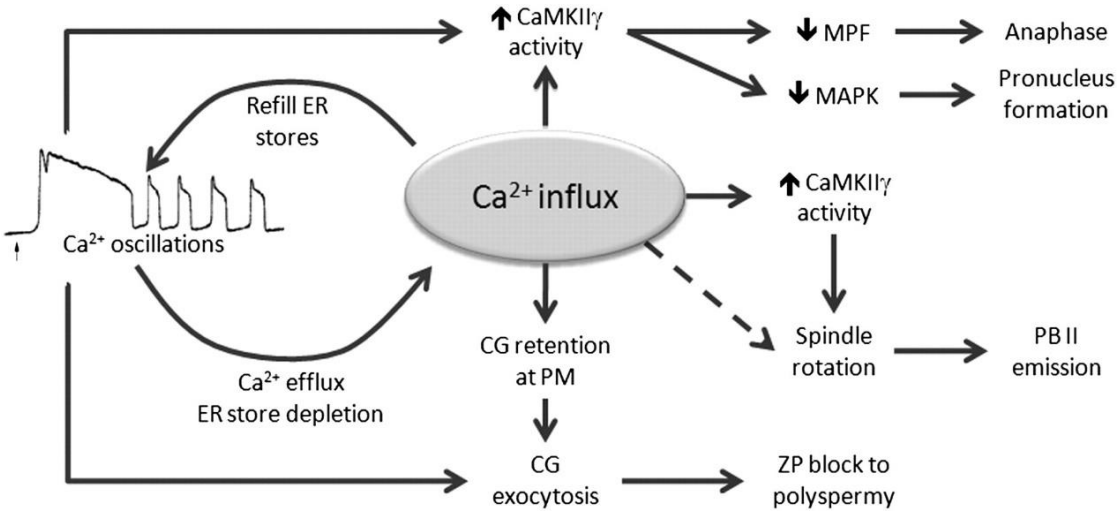
These channels/mechanisms play a pivotal role in sustaining Ca^{2+} homeostasis and supporting oscillations, IP_3R modifications and ER re-organization (Wakai and Fissore, 2013). Moreover, TRP channels which are widely expressed in non-excitable cells may also have important roles in modulating Ca^{2+} influx (Gees *et al.*, 2010). Of note, some TRP channels can be sensitized by Ca^{2+} or by PKC-dependent phosphorylation. For instance, PLC generated diacylglycerol activates canonical TRPC channels at fertilization (Hardie, 2007), which can be detected at the egg PM.

Mitochondria also contribute to shape intracellular Ca^{2+} rises during oscillations (Duchen, 2000). These organelles can uptake Ca^{2+} into their matrix, thereby alleviating the overall cytosolic Ca^{2+} load (Rizzuto *et al.*, 1998). However, the uptake of Ca^{2+} does not seem to be their main function in terms of Ca^{2+} homeostasis, as inhibition of mitochondrial function causes a sustained increase in Ca^{2+} in mouse eggs (Liu *et al.*, 2001), but does not immediately terminate sperm-initiated oscillations (Dumollard *et al.*, 2004). Instead, the Ca^{2+}-driven ATP output may be the mitochondria's most critical contribution to Ca^{2+} homeostasis in MII eggs, as ATP production maintains SERCA activity, which is required for maintaining $[Ca^{2+}]_{ER}$ and sustaining sperm-triggered Ca^{2+} oscillations (Kline and Kline, 1992).

1.3 IP_3/Ca^{2+} signaling accompanies molecular alterations during oocyte activation

Upon sperm penetration at fertilization, each sperm-induced Ca^{2+} rise is accompanied by a parallel increase in Ca^{2+}/Calmodulin dependent protein kinase II (CaMKII) activity (Backs *et al.*, 2010). Subsequently, this inhibits Emi2 and leads to the activation of APC/C (Medvedev *et al.*, 2014) (Figure 7). Thereafter, similar to the metaphase I-anaphase I transition, APC/C activation results in destruction of MPF, as well as mitogen-activated protein kinase (MAPK), and the release of separase (Terret *et al.*, 2003), which triggers resumption of meiosis (Ducibella and Fissore, 2008; Sanders and Swann, 2016) (Figure 7). Similarly, protein kinase C activation which is implicated in cell-cycle resumption, occurs with a temporal pattern similar to the timing of Ca^{2+} oscillations (Halet, 2004).

Figure 7. Ca^{2+} signaling triggers MPF related molecular pathway during oocyte activation. Figure reprinted with permission. (Miao *et al.*, 2012)



1.4 Deregulation of Ca^{2+} signaling and female infertility

Deregulation of Ca^{2+} signaling of excitable and non-excitable cells, accounts for the incidence of a large number of human diseases, as diverse as neural diseases (Alzheimer's disease, bipolar disorder), cardiac diseases, such as atrial arrhythmias, ventricular hypertrophy, congestive heart failure, and cancer, diabetes, liver cholestasis, pancreatitis etc. (Carafoli and Brini, 2007; Feske, 2007; Berridge, 2012b, 2016; Derouiche *et al.*, 2013; Mikoshiba, 2015). Moreover, reproductive failures may also relate to the deregulation of Ca^{2+} signaling of the oocyte, a representative example of non-excitable cells.

1.4.1 Meiotic maturation resistance

Abnormal Ca^{2+} signaling is observed in a maturation deficient mouse model, LT/Sv mice (Archacka *et al.*, 2008). Oocytes from LT/Sv mice show an abnormal regulation of meiotic maturation, many of which are unable to complete the first meiotic division and become arrested at the MI stage. The origin of MI arrest of LT/Sv oocytes is suggested to be of a cytoplasmic nature and could be overcome by nuclear transfer (Hoffmann *et al.*, 2012). However, only little is known regarding this treatment in patients with maturation resistant oocytes.

In assisted reproductive technologies (ART) cycles, approximately 11% and 4% of the harvested oocytes are at prophase I and MI stage respectively (Reichman *et al.*, 2010; De Vincentiis *et al.*, 2013); most of these are meiotically competent and resume meiosis during subsequent *in vitro* culture (Combelles *et al.*, 2002; Heindryckx *et al.*, 2008). However, approximately 8.6% of infertility patients produce at least one maturation resistant oocyte, which fails to resume meiosis *in vivo*, even after subsequent *in vitro* incubation (Bar-Ami *et al.*,

1994). Interestingly, this maturation resistance occurs repeatedly in 0.1-1% of the patients with all or most of the collected oocytes in immature stage. They are mostly arrested at the MI stage and resistant to further spontaneous meiotic maturation despite prolonged *in vitro* culture (Harrison *et al.*, 2000; Bergere *et al.*, 2001; Levran *et al.*, 2002; Neal *et al.*, 2002; Beall *et al.*, 2010; Heindryckx *et al.*, 2011a).

A dozen of case reports and case series describe patients with recurrent oocyte maturation resistance (Table 1). The key clinical features of these patients include primary infertility, repetitive production of mostly immature oocytes, inability of IVM to stimulate further meiosis and mostly failed fertilization following ICSI (Beall *et al.*, 2010). Highly aberrant spindle-chromosome complex configurations are observed in meiotic maturation resistant oocytes at the MI stage in some case reports (Windt *et al.*, 2001; Combelles *et al.*, 2003) and in 4 cases in our Department who were showing all immature oocytes at retrieval (Heindryckx *et al.*, 2011b). Moreover, a study has shown that no pregnancies could be achieved in patients presenting with more than 25% of maturation resistant oocytes in an assisted reproductive cycle (Bar-Ami *et al.*, 1994). Currently, the origin and cytoplasmic feature of human maturation resistant oocytes remains unclear.

Table 1 Clinical outcomes of patients with a high number of maturation resistant oocytes

TABLE 1

Case reports describing infertility patients with the syndrome of oocyte maturation failure.

OMF type	No. of patients	Country	Results	Reference
I	1	Israel	GV arrest	60
II	1	Germany	MI arrest	61
	1	UK	MI arrest. Initially GV then with IVM progressed to and arrested at MI. Oocytes remained arrested at MI despite incubation in gonadotropin.	62
	2	Australia	MI arrest, underwent IVM and ICSI without further maturation or fertilization	63
	1	France	MI arrest. Unsuccessful IVM. Patient with a family history significant for three of four sisters with infertility.	64
	1	Canada	MI arrest. Unsuccessful IVM. Ultrastructural analysis demonstrated irregular chromatin condensation, disrupted spindle formation, and distorted and/or stunted microvilli protruding to the perivitelline space	65
	3	Israel	MI arrest. Failed IVM.	60
III	1	Israel	MII arrest	60
IV	3	Israel	Mixed arrest (one GV + MI, one GV + MII, one MI + MII)	60
	3	Israel	One patient with GV + MI arrest, failed IVM. Two patients (sisters) with mixed MI and MII arrest, fertilized with pronuclei observed but no polar bodies. All had an abnormal chromosomal number.	66
	2	USA	One patient with GV + MI arrest. IVM with progression to MII in 3/16 oocytes. ICSI performed without fertilization. Ultrastructural analysis demonstrated chromosomal aberrations and disrupted spindle formation. One patient underwent four cycles with between 40% and 100% immature oocytes retrieved. Mixed GV (8%) + MI (65%) arrest with MII (22%). IVM performed without success. ICSI performed on MII with fertilization (total four embryos and two transfers) without pregnancy.	67

Note: IVM = in vitro maturation; ICSI = intracytoplasmic sperm injection.

Beall. Oocyte maturation failure: a syndrome of bad eggs. Fertil Steril 2010.

Table reprinted with permission. (Beall *et al.*, 2010).

1.4.2 Failed fertilization and assisted oocyte activation (AOA)

Intracytoplasmic sperm injection (ICSI) results in fertilization rates between 70% and 80% when viable sperm is present in the ejaculate or testis (Palermo *et al.*, 2009). While ICSI has overcome many infertility defects, in particular male infertility, it does not completely eliminate fertilization failure. In some ICSI cases, with a frequency ranging from 3 to 5% (Esfandiari *et al.*, 2005), complete fertilization failure or abnormal low fertilization still occurs (Devroey *et al.*, 1995; Flaherty *et al.*, 1998; Yanagida, 2004). The possible etiologies underlying total fertilization failure are complex (Swain and Pool, 2008) and may relate to cycle-specific parameters, oocyte yield and quality, availability of motile spermatozoa and/or severity of sperm defects (Esfandiari *et al.*, 2005), but deficiencies in the oocyte activation mechanism account for the primary cause. Additionally, ooplasmic dysmaturity in relation to the achieved nuclear maturation marked by the extrusion of the first polar body can result in failure to fertilize.

More uncommon are cases where the spermatozoa partially or completely lack the specific oocyte activating factor (Kashir *et al.*, 2010; Neri *et al.*, 2014), leading to fertilization failure after ICSI (Kovacic and Vlaisavljevic, 2000). Under some circumstances, this ICSI failure has been successfully overcome by the application of the AOA technology, by manipulating intracellular Ca^{2+} influx with a variety of physical, mechanical or chemical activation stimuli, such as electrical pulses, modified ICSI procedure, or calcium ionophores and strontium chloride ($SrCl_2$) (Vanden Meerschaut *et al.*, 2014). Among all these, the most widely used artificial activating agents for human oocytes are the Ca^{2+} selective ionophores, ionomycin and calcimycin (also known as A23187) (Heindryckx *et al.*, 2008; Ebner *et al.*, 2012).

Despite the availability of different AOA technologies, some laboratories report lower fertilization and pregnancy rates after using different AOA protocols (Ebner *et al.*, 2012; Montag *et al.*, 2012) compared to others (Heindryckx *et al.*, 2008), or no differences in fertilization rate after the application of AOA compared to routine ICSI (Borges Jr. *et al.*, 2009). The differences in the efficiency of AOA protocols might be due to the patients' fertility background, the lack of diagnostic tests or the specific AOA protocols used (Vanden Meerschaut *et al.*, 2014).

The variability in protein source, ions and EDTA present in the various commercial human embryo culture media could attribute to the altered embryonic development potential (Morbeck *et al.* 2014a; Morbeck *et al.* 2017). The variation in the composition of protein supplements in the medium influences the blastocyst developmental potential in mouse (Morbeck *et al.* 2014b). Moreover, fertilizing oocytes in medium with reduced concentration of Mg^{2+} (0.2mM), significantly improved blastocysts rates in both mouse and human (Herrick *et al.*, 2015). Furthermore, the concentration of the membrane-impermeable metal ion chelator could also

influence the treatment outcomes, for instance EDTA impairs embryonic developmental potential at a higher concentrations (>5mM) (Berridge *et al.*, 2000). Investigations are required to verify the effect of specific components of the embryo culture medium. Currently, the concentration of Ca^{2+} ([Ca^{2+}]) in the culture medium has not been taken into account during the application of AOA technology to overcome failed fertilization.

Moreover, $SrCl_2$ is widely used to induce very efficiently artificial activation of mouse eggs following somatic cell nuclear transfer or round spermatid injection (Loren and Lacham-Kaplan, 2006). Although successful pregnancies were reported after using Sr^{2+} during AOA, in couples with low fertilization rates from several groups (Yanagida *et al.*, 2006; Kyono *et al.*, 2008; Kim *et al.*, 2014) (Table 2), the efficiency of Sr^{2+} as an activating agent for human oocytes is still under debate. The exact mechanism by which Sr^{2+} induces activation in human oocytes remains unclear. One possible explanation is that Sr^{2+} moves into the oocyte down the concentration gradient, causing Ca^{2+} release from the ER through the IP_3 receptor (Zhang *et al.*, 2005). Recently, it was found that the TRP channel, subfamily V, member 3 (vanilloid 3) (TRPV3) mediates Sr^{2+} induced oocyte activation (Carvacho *et al.*, 2013) and Ca^{2+} influx induced by 2-aminoethoxydiphenyl borate (2-APB) in mouse oocytes (Lee *et al.*, 2016). Generally, TRP channels are modulated by a variety of stimuli and ligands (Ramsey *et al.*, 2006), including various natural compounds like carvacrol, thymol and eugenol (Xu *et al.*, 2006). Remarkably, activation of TRPV3 channels by 2-APB and carvacrol promotes Ca^{2+} entry and provokes mouse oocyte activation without temperature sensitization, whereas, the absence of $TRPV_3$ channels in mouse oocytes failed to provoke Sr^{2+} induced activation (Carvacho *et al.*, 2013). However, it is still unknown if Sr^{2+} induces Ca^{2+} rise efficiently in human oocytes and through which channels this is mediated.

Table 2. Pregnancy outcomes of patients with fertilization failure following AOA treatment by using Sr^{2+}

Fertilization failure	Patients (n)	Protocol	Pregnancy outcome	Reference
Complete failed fertilization Low fertilization rates after ICSI and AOA	8	Post ICSI (1h) incubation in 10mM $SrCl_2$ for 30 min	5 successful pregnancies 8 livebirths	Kim *et al.*, 2014
Complete failed fertilization Low fertilization rates (0% -16.7%)	6	$SrCl_2$ incubation following ICSI	3 successful pregnancies 3 livebirths	Chen *et al.*, 2012
Frozen-thawed testicular spermatozo	1	Post ICSI (30min) incubation in 10mM of $SrCl_2$ for 1h	1 successful pregnancy 1 livebirth	Kim *et al.*, 2012
Globozoospermia	1	Post ICSI incubation in 10mM $SrCl_2$ for 10 min	1 successful pregnancy 2 livebirths	Yang *et al.*, 2012
Repeated fertilization failure	9	Post ICSI incubation in 10mM $SrCl_2$ for 1h	6 successful pregnancies 5 livebirths	Kyono *et al.*, 2008
Repeated low fertilization rates (0%-33.3% from 8 ICSI cycles)	1	Post ICSI (1h) incubation in 10mM of $SrCl_2$ for 30 min	2 successful pregnancies (fresh and cryopreserved) 1 livebirth	Yanagida *et al.*, 2006

1.4.3 **Oocyte post-ovulatory ageing and reproductive ageing**

An optimal window exists in fertilization of the MII stage oocyte, which is generally within 10 hours of ovulation in mammals (Marston and Chang, 1964; Tarín *et al.*, 1999). In the event that no fertilization occurs within this time frame, oocytes experience deterioration in quality referred to as post-ovulatory oocyte ageing (Lord and Aitken, 2013). It occurs both *in vivo* and *in vitro*, either in the oviduct of the female reproductive tract, or in the *in vitro* culture media in an assisted reproduction setting. At fertilization, Ca^{2+}-signaling is deregulated in post-ovulatory aged oocytes, showing a higher frequency and lower amplitude in Ca^{2+} oscillatory patterns (Kolker *et al.*, 2003; Takahashi *et al.*, 2009). As such, they activate pathways of apoptosis, fragmentation, and cell death, instead of supporting fertilization and normal embryonic development (Gordo *et al.*, 2002; Takahashi *et al.*, 2009).

Post-ovulatory aged oocytes can still serve as valuable material for rescue-intracytoplasmic sperm injection to produce offspring in the clinic (Nagy *et al.*, 1993) and establish embryonic stem cell lines from somatic cell nuclei by nuclear transfer (Thuan *et al.*, 2010). These oocytes exhibit numerous aberrations in their cell biology, including a decrease in critical cell cycle factors including MPF and MAPK (Kikuchi *et al.*, 2002), mitochondrial dysfunction (Tatone *et al.*, 2011; Lord and Aitken, 2013), spindle abnormalities and losses of chromosomal integrity (Wakayama *et al.*, 2004). This has also been associated with decreased fertilization capacity (Marston and Chang, 1964; Ben-Rafael *et al.*, 1986; Badenas *et al.*, 1989), poor embryo quality (Yanagida *et al.*, 1998; Lord *et al.*, 2013) and abnormalities in offspring (Tarín *et al.*, 1999).

Reproductive ageing is a process that is distinct from post-ovulatory ageing, which relates to decreased oocyte quality in patients with advanced age. The reduced ovarian reserve and an increased rate of chromosomal aberrations leads to an increased risk of miscarriages and aneuploidy of older patients (Bartmann *et al.*, 2004; Wright *et al.*, 2008). Meiotic errors during MI stage represent the leading cause of pregnancy loss and congenital defects (Hunt and Hassold, 2008), and their incidence is strongly influenced by maternal age (Hook *et al.*, 1981; Nagaoka *et al.*, 2011). However, Ca^{2+} signaling alterations have not been demonstrated in reproductive ageing.

In vitro maturation (IVM) is an emerging procedure incorporated into the world of assisted reproductive technologies. Fertilizing IVM oocytes represents a cheaper and safer approach compared to routine *in vitro* fertilization, particularly for women suffering from polycystic ovarian syndrome. However, in these cases, an impaired ability of generating Ca^{2+} at fertilization (Cheung *et al.*, 2000; Mann *et al.*, 2010) has been observed in both mouse and human. The impact of post-ovulatory and reproductive ageing on the Ca^{2+} signaling of IVM oocytes has also not been elucidated.

1.5 Methodologies for investigating the Ca^{2+} signal

To date, there are a number of viable tools to investigate certain Ca^{2+} signaling related parameters. For analysing the extracellular Ca^{2+} amount, the concentration of Ca^{2+} supplemented in the embryo culture medium could be measured by direct potentiometry with ion-selective electrodes, similar to Ca^{2+} measurements in serum, plasma, whole blood, and other biological fluid samples (Sena and Bowers, 1988). The measurement range of Ca^{2+} selective electrodes is 3µM to 1M. The pH monitor and the calibration of the electrode is critical to the accuracy of the measurement. The colorimetric assay kit is also used for quantification of Ca^{2+} present in cell lysates and other biological samples. This method can detect Ca^{2+} concentrations from 0.1 to 25mM. When analyzing the total amount of calcium, atomic absorption spectrometry and polarized Energy Dispersive X-Ray Fluorescence (XRF)

systems are available. The accuracy of XRF tech is 0.001M, while the detection limit is around 0.1mM.

To measure intracellular Ca^{2+}, Ca^{2+}-imaging is commonly applied. Ca^{2+}-imaging is useful for measuring calcium signals in cultured cells. This technique takes advantage of Ca^{2+} indicator dyes, which are BAPTA-based organic molecules that change their spectral properties in response to the binding of Ca^{2+} ions. Ca^{2+} indicator dyes fall into two categories, ratio-metric dyes like Fura-2 and Indo-1 and single-wavelength dyes like Fluo-4. For our study, the ratio-metric dye Fura-2 is used, it has an emission peak at 505 nM and changes its excitation peak from 340 to 380 nm in response to Ca^{2+} binding, allowing the concentration of intracellular Ca^{2+} to be determined from the ratio of fluorescence excitation. The main advantage of this ratio-metric dye over single wavelength probes is that the ratio signal is independent of the dye concentration, illumination intensity, and optical path length allowing the concentration of intracellular Ca^{2+} to be determined independently of these artifacts (Barreto-Chang and Dolmetsch, 2009).

Nowadays, for couples experiencing failed fertilization after ICSI, heterologous ICSI models and mouse oocyte Ca^{2+} analysis has been successfully used to reveal activation deficiencies and determine whether they are sperm- or oocyte-related (Rybouchkin *et al.*, 1996; Araki *et al.*, 2004; Heindryckx *et al.*, 2005). The Ca^{2+} oscillatory pattern analysis by Ca^{2+}-imaging following heterologous ICSI has the strength to reveal subtle deficiencies in underlying sperm-borne activation deficiency responsible for low or failed fertilization after conventional ICSI.

Non-invasive particle velocimetry can also evaluate the intracellular Ca^{2+} signals, this technique detects small cytoplasmic movements of the oocytes synchronous with and dependent on Ca^{2+} oscillations. This technique has been applied on failed fertilized oocytes (Swann *et al.*, 2012). However, the detrimental effect of frequent light exposure (every 10 second) has to be accessed before the application. Moreover, in oocytes, multiple Ca^{2+} signaling pathways may be activated by one stimulus. Therefore, it is difficult to identify the proportion of Ca^{2+} released from ER and the amount of Ca^{2+} influx through PM channels. The utility of selective inhibitors of certain Ca^{2+} channels may serve as a tool to define the origin of Ca^{2+}, for example, thapsigargin, which blocks a class of enzymes known by the acronym SERCA, and thus inhibits the ability of the cell to pump calcium into the SR/ER which causes these stores to become depleted (Kobrinsky and Kirchberger, 2001).

Chapter 2

2. Aims of the thesis

This thesis investigates the Ca^{2+} signaling alterations in oocytes showing maturation resistance, oocytes after specific AOA treatments and oocytes undergoing post-ovulatory ageing or reproductive ageing.

1) **Nuclear and cytoplasmic (Ca^{2+}-signaling) abnormalities in maturation resistant oocytes**

In ART cycles, maturation resistance of oocytes can occur repeatedly in 0.1-1% of patients. In such cases, all or most of the oocytes retrieved are immature and further resistant to meiotic progression and pregnancies can rarely be achieved. Oocytes from LT/Sv mice also show an abnormal regulation of meiotic maturation, many of which are unable to complete the first meiotic division and become arrested at the MI stage. It has been suggested that the origin of MI arrest of LT/Sv oocytes is of a cytoplasmic nature (Hoffmann *et al.*, 2012).

In the first part of the thesis, we investigated the nuclear and cytoplasmic status of maturation resistant oocytes from patients presenting with >40% immature oocytes at retrieval. Oocytes obtained from this patient group are likely to be resistant to meiotic maturation. The ICSI treatment outcomes of these patients were followed-up. We further aimed to perform an in-depth evaluation of Ca^{2+}-signaling alterations in the meiosis maturation resistant mouse model **(Chapter 3)**.

2) **Evaluation of the effect of specific activating agents and culture components on the efficiency of AOA through Ca^{2+}-signaling analysis**

Following ICSI treatment, 1-5% of patients experience complete fertilization failure. Oocyte-related ICSI failure may be successfully overcome by application of AOA. It is commonly performed by manipulating intracellular Ca^{2+} influx with chemical activation stimuli, for instance ionomycin and Sr^{2+}.

As variations in the composition of commercial culture media have been shown to affect embryo developmental potential (Morbeck *et al.* 2014a; Morbeck *et al.* 2017), varying media supplements and components may also influence the AOA outcome. In this part of the thesis, we assessed the effect of external factors (concentrations of ionomycin and Ca^{2+}) present in culture media on the Ca^{2+} signal during AOA **(Chapter 4)**.

Several successful pregnancies have been obtained after using Sr^{2+} during (Yanagida *et al.*, 2006; Kyono *et al.*, 2008; Kim *et al.*, 2014). However, the efficiency of Sr^{2+} as an activating agent for human oocytes is still under debate. As such, we further aimed to verify the effectiveness of Sr^{2+} for human oocyte activation (**Chapter 5**).

3) Alterations in Ca^{2+} oscillatory patterns in aged mouse oocytes

Oocyte ageing, due to advanced female age (reproductive oocyte ageing) and prolonged *in vitro* culture (post-ovulatory ageing) are both associated with decreased fertilization capacity and poor embryo quality (Marston and Chang, 1964; Ben-Rafael *et al.*, 1986; Badenas *et al.*, 1989). The Ca^{2+} oscillatory pattern of reproductive-aged oocytes has not been investigated. Therefore, we aimed to evaluate the differences between post-ovulatory and reproductive-aged mouse oocytes with a focus on Ca^{2+} oscillatory pattern analysis and subsequent embryo development, following Sr^{2+} exposure **(Chapter 6)**.

Chapter 3

3. Patients with a high proportion of immature and meiotically resistant oocytes experience defective nuclear oocyte maturation patterns and impaired pregnancy outcomes

Objective

Patients presenting with an abnormally high number of immature oocytes at retrieval are more likely to exhibit maturation resistant oocytes. In the first study of this thesis, we investigated the spindle-chromosome abnormalities of immature oocytes following in vitro culture, from patients showing >40% of immature oocytes at retrieval. Pregnancy outcomes were additionally estimated in these patients based on the presence of MR oocytes.

Conclusion

A high percentage of abnormal spindle-chromosome configurations was observed in maturation resistant and IVM oocytes from patients showing >40% of immature oocytes at retrieval. Cumulative pregnancy rates and live birth rates were significantly lower in these patients when compared to controls, who showed a normal number of mature oocytes at retrieval. The treatment strategy and the utility of IVM in patients with a high proportion of immature and MR oocytes warrant clinical concern.

Patients with a high proportion of immature and meiotically resistant oocytes experience defective nuclear oocyte maturation patterns and impaired pregnancy outcomes

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Short title: Outcomes of patients with immature and maturation resistant oocytes

Abstract

Patients presenting with an abnormally high number of immature oocytes at retrieval are more likely to exhibit maturation resistant oocytes. However, the clinical relevance of such events remains unknown. We investigated the nuclear maturation competence of immature oocytes from patients showing >40% of collected immature oocytes (Study group) and Controls, in which a normal number of mature oocytes (≥60%) was retrieved. Following in-vitro culture, oocytes were classified as maturation resistant and in-vitro matured (IVM). Treatment outcomes were evaluated in Study and Control groups based on the presence of maturation resistant oocytes. Overall, similarly high spindle and chromosome abnormality rates were observed in maturation resistant oocytes from both Study and Control groups. IVM oocytes from Study group revealed a significantly higher percentage of misaligned chromosomes compared with Controls (P<0.05). Remarkably, Study patients with at least one maturation resistant oocyte, showed significantly reduced cumulative pregnancy and live birth rates compared with Control maturation resistant patients (P<0.05). To further investigate the aetiology, we applied a maturation resistant mouse model and revealed defective Ca^{2+}-signaling of maturation resistant oocytes at germinal vesicular breakdown and parthenogenetic activation. In conclusion, appropriate treatment strategies, including clinical utilization of IVM oocytes from the Study group patients warrant further investigation.

KEYWORDS: Ca^{2+}-signaling, chromosome misalignment, meiosis resistant, immature oocytes, nuclear maturation, spindle abnormality.

Introduction

Infertility is a complex pathophysiological disorder. It can be caused by various factors, ranging from hormonal imbalances to genetic alterations and even environmental influence (McCallie et al., 2017). The Human Fertilization and Embryology Authority (HFEA) reported that one out of six couples are struggling with infertility, and the origins are equally distributed between male and female (Bracewell-Milnes et al., 2017). Nowadays, assisted reproductive technology (ART) is used primarily for infertility treatments to achieve a pregnancy.

Ovarian stimulation protocols, as part of standardized infertility treatments, predominately generate mature metaphase II (MII) oocytes. However, approximately 15-20% of oocytes retrieved after ovarian stimulation for conventional IVF are immature (Reichman et al., 2010). Previous reports have documented that most of these oocytes maintain meiotic competence and may resume meiosis during extended in-vitro culture (Combelles et al., 2002, Heindryckx et al., 2007). Nevertheless, approximately 8.6% of subfertile patients produce at least one oocyte resistant to meiotic maturation. Such maturation resistant oocytes fail to resume meiosis even following subsequent in-vitro culture (Bar-Ami et al., 1994). Moreover, in 0.1-1% of cases all or most of the recovered oocytes are immature and exhibit complete oocyte maturation arrest (Archacka et al., 2008). These oocytes are mostly arrested at the metaphase I (MI) stage and show resistance to further meiotic maturation even following prolonged in-vitro culture in standardized conditions (Harrison et al., 2000, Bergere et al., 2001, Levran et al., 2002, Neal et al., 2002, Beall et al., 2010, Heindryckx et al., 2011).

Although complete failure of human oocytes to resume meiosis is rare (Levran et al., 2002), previous reports have indicated that in cases where the percentage of maturation resistant oocytes was greater than 25%, IVF outcomes were significantly reduced and no pregnancies could be achieved (Bar-Ami et al., 1994, Levran et al., 2002). Patients showing an abnormally high percentage of immature oocytes at retrieval represent a great possibility of exhibiting maturation resistant oocytes, however, the oocyte maturation competence and the pregnancy outcomes of these patients currently remain largely unknown. Both nuclear as well as cytoplasmic, structural and biochemical changes provide oocytes with the capacity to progress through meiosis (Levran et al., 2002, Fulka Jr. et al., 1998). Highly aberrant spindle-chromosome complex configurations have been observed in maturation resistant oocytes obtained from patients, for which no mature oocytes were retrieved (Windt et al., 2001, Combelles et al., 2003, Heindryckx et al., 2011).

Moreover, evidence from a subfertile mouse model demonstrated that in-vitro cultured maturation resistant oocytes display aberrant spindles and an impaired ability to exhibit Ca^{2+}-transients after fertilization (Archacka et al., 2008). The LT/Sv female mice show a high number of immature oocytes following ovarian stimulation, most of which are arrested at MI

stage (Eppig, 1978, Hupalowska et al., 2008), as observed in human maturation resistant patients (Beall et al., 2010). The LT/Sv maturation resistant oocytes can become activated spontaneously and undergo embryonic development forming teratoma in the ovary (Eppig, 1978). The cause of the maturation resistant oocytes from LT/Sv mice was reported to be of cytoplasmic origin (Hoffmann et al., 2012), and related with the over-activation of spindle-assembly-checkpoint (SAC) proteins (Ciemerych and Kubiak, 1998; Hoffmann et al., 2012; Hupalowska et al., 2008). However, further research into the exact mechanisms governing oocyte maturation is essential for defining precise deficiencies impeding meiotic competence (Levran et al., 2002).

Successful oocyte maturation and activation are mediated by inositol 1,4,5-trisphosphate receptors (IP_3Rs) generated characteristic Ca^{2+}-oscillations in oocyte, either released spontaneously or triggered by the sperm factor phospholipase C zeta respectively (Saunders et al., 2002, Tesarik, 2002, Ramadan et al., 2012, Wakai et al., 2013). In LT/Sv mice, the Ca^{2+}-oscillatory ability and the nuclear maturation normality of collected fresh MI arrested oocytes remains unclear. Analysis of Ca^{2+}-signaling patterns of in-vitro cultured and in-vivo collected maturation resistant oocytes in this mouse model may provide additional insights into the underlying processes of meiotic resistance in human. Shedding light on the physiology of oocyte maturation in human may allow for more effective diagnostic approaches.

In this study, we compared the nuclear maturation competence of immature oocytes obtained from patients presenting with a high rate (>40%) of immature oocytes at retrieval (Study group), with immature and in-vivo matured oocytes retrieved from patients showing a normal number (≥60%) of collected mature oocytes (Control group). Following in-vitro culture, immature oocytes from Study and Control group were classified for spindle-chromosome configuration analysis, based on their maturation status: maturation resistant and in-vitro matured (IVM). Moreover, fertilization rates and pregnancy outcomes were evaluated in both groups based on the presence of maturation resistant oocytes observed after in-vitro culture. Finally, we used a LT/Sv maturation resistant mouse model to assess the Ca^{2+}-signaling of maturation resistant oocytes during oocyte maturation and at parthenogenetic activation.

Materials and methods

All chemicals and reagents were purchased from Sigma Chemical Co. (Diegem Belgium), unless otherwise indicated.

Ethical approval

The study was approved by the local Ethical Committee of Ghent University Hospital, Belgium (reference numbers: 2009/130 approved on March 31st, 2009, 2010/182 approved on April 21st, 2010 and 2010/808 approved on January 20th, 2011). 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) on December 12th, 2011.

Source of human oocytes

Patients (25-44 years old) undergoing ICSI treatment at the Ghent University Hospital, between November 2011 and May 2014, were included in this study. Patients undergoing a hormone stimulated cycle were administrated with a GnRH agonist (Decapeptyl; Ferring) or antagonist (Cetrotide;Merck Serono). Ovarian stimulation was performed by administering hMG (Menopur; Ferring) or recombinant FSH (Gonal-F; Merck Serono) at a dose of 112.5-300IU daily and ovulation was induced with 5000IU hCG (Pregnyl;MSD). Oocytes were enzymatically denuded by brief exposure to 80IU/ml hyaluronidase (Irvine Scientific), followed by mechanical denudation prior to ICSI. Nuclear status was assessed and classified as germinal vesicle (GV) (presence of a GV structure), MI (absence of both a polar body and a GV structure) or MII stage (presence of a polar body and absence of a GV structure). Donated GV oocytes were further cultured in medium 199, supplemented with 10ng/ml epidermal growth factor, 1mg/ml estradiol, 10mIU/ml recombinant FSH, 0.5mIU/ml hCG, 1mM l-glutamine, 0.3mM sodium pyruvate, 0.8% (v/v) human serum albumin (Red Cross, Belgium), 100IU/ml penicillin G and 100mg/ml streptomycin sulphate at 37°C in 6% CO_2, 5% O_2. Based on 1st polar body extrusion, GV oocytes which processed to MII stage after 24h or 48h of in-vitro culture were defined as GV-MII 24h or GV-MII 48h. Oocytes collected at MI stage were cultured in Sydney IVF cleavage medium (Cook Ireland Ltd), in which oocytes reached to MII stage following 3h or 24h of in-vitro culture were identified as MI-II 3h or MI-II 24h. GV oocytes that matured to the MI stage and showed resistance to progress to MII stage after 48 hours of culture were defined as GV-MI 48h. Donated MI oocytes which failed to extrude 1st polar body within 24h of in-vitro culture in Cook Cleavage medium were defined as MI-24h. For treatment of the patients, ICSI was performed by standardized techniques in normal in-vivo matured MII oocytes. Fertilization (oocytes showing 2PN and a second polar body) rate was assessed 14-18h post injection.

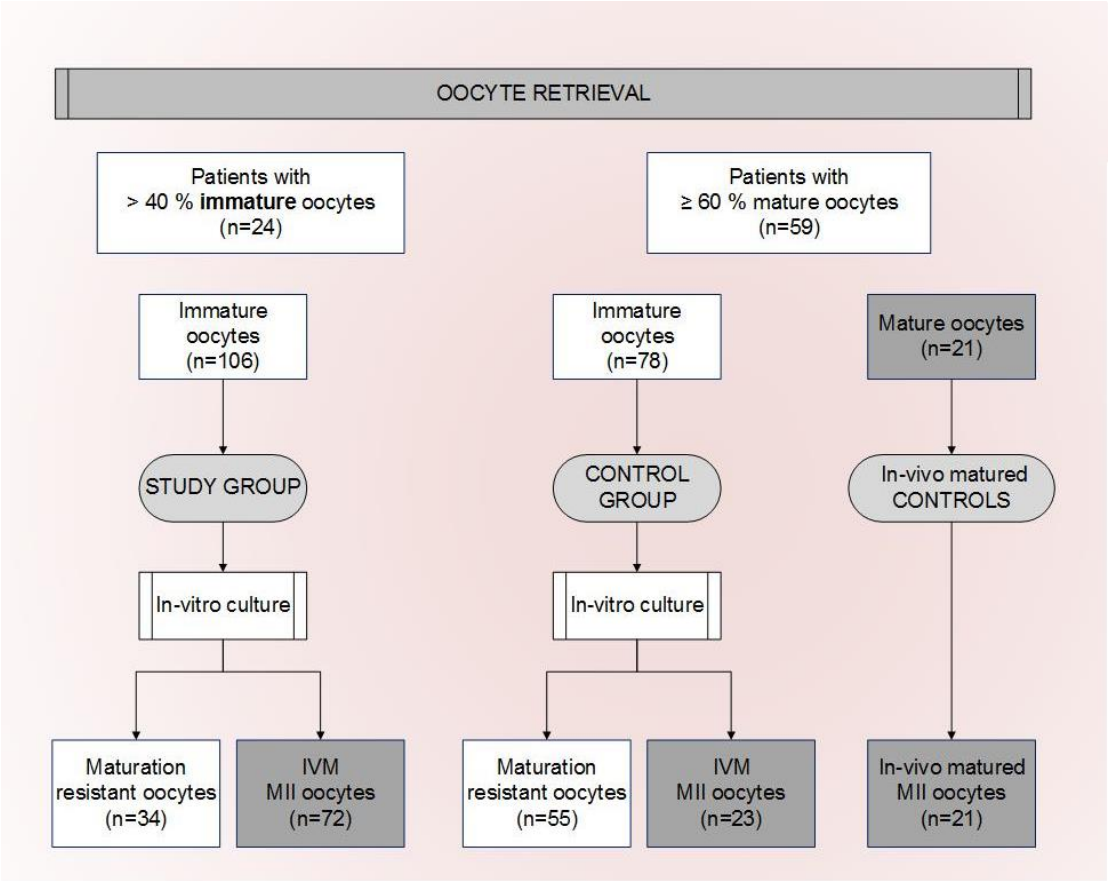
Embryos were vitrified/warmed using a closed vitrification protocol (Irvine Scientific). Embryos were initially incubated in a 50ml drop of Sydney IVF Gamete buffer medium (GB) (Cook Ireland Ltd) for 1min at 37°C and for 1min at room temperature (RT). The GB drop was then merged for 2min with an adjacent 50ml drop of equilibration solution (ES) containing 7.5% ethylene glycol and 7.5% DMSO. The resulting drop was further merged with a fresh drop of ES for 2min before incubating the embryos in a separate 50ml ES drop for 10min at RT. Then, embryos were sequentially incubated at RT in 4 drops of 25ml vitrification solution containing 15% (v/v) ethylene glycol, 15% (v/v) DMSO and 0.5M sucrose for 60–90s in total. The embryos were finally loaded onto High Security straws (Cryo Bio System) with a minimal

volume and the straws were thermo sealed before being plunged into liquid nitrogen. For warming, embryos were immediately placed in a 500ml drop of thawing solution containing 1M sucrose for 1min at 37°C and then for 1min at RT. Embryos were subsequently placed in a 50ml drop of thawing solution for 1min at RT and then in two 50ml drops of dilution solution containing 0.5M sucrose for 2min each. Finally, embryos were incubated in three 50ml drops of washing solution for 3min each. Following the warming procedure, embryos were cultured in Cook Blastocyst medium at 37°C under 6% CO_2, 5% O_2 until transfer.

The treatment main end-points were fertilization rate, clinical pregnancy rate, miscarriage rate and live birth rate in this oocyte retrieval. The clinical pregnancy was defined as the visualization of at least one gestational sac by ultrasound confirmation at about 6-8 weeks gestational age. Additionally, cumulative pregnancy rate, miscarriage rate and live birth rates were recorded from the subsequent stimulations following the oocyte retrieval that was investigated.

Oocytes (n=106) from a total of 24 patients showing an abnormally high percentage of immature oocytes (>40%) at oocyte retrieval were included in Study group and classified based on their maturation stage: maturation resistant oocytes (MI-24h) (n=34) and IVM oocytes (13 GV-MII 24h, 20 MI-II 3h and 39 MI-II 24h) (n=72) (Supplementary Figure 1). Patients with 40% or more immature oocytes retrieved during an ICSI cycle represented 5% of the assisted reproductive population in our center (based on the database from Jan 2003 to September 2011 in Ghent University Hospital). For defining the Controls, we allocated oocytes (n=99) from 59 patients presenting with a normal number (≥60%) of mature oocytes at oocyte retrieval, included 55 maturation resistant (9 GV-MI 48h and 46 MI-24h) and 23 IVM oocytes (8 GV-MII 24h and 6 MI-II 3h, 9 MI-II 24h) from 54 patients, as well as 14 donated fresh in-vivo matured MII oocytes obtained from a cancelled treatment and 7 in-vivo matured MII oocytes showing visible aggregates of smooth ER clusters (SERs) from 4 patients (Supplementary Figure 1).

Supplementary Figure 1. Flow chart showing the inclusion of human immature and mature oocytes in the study.



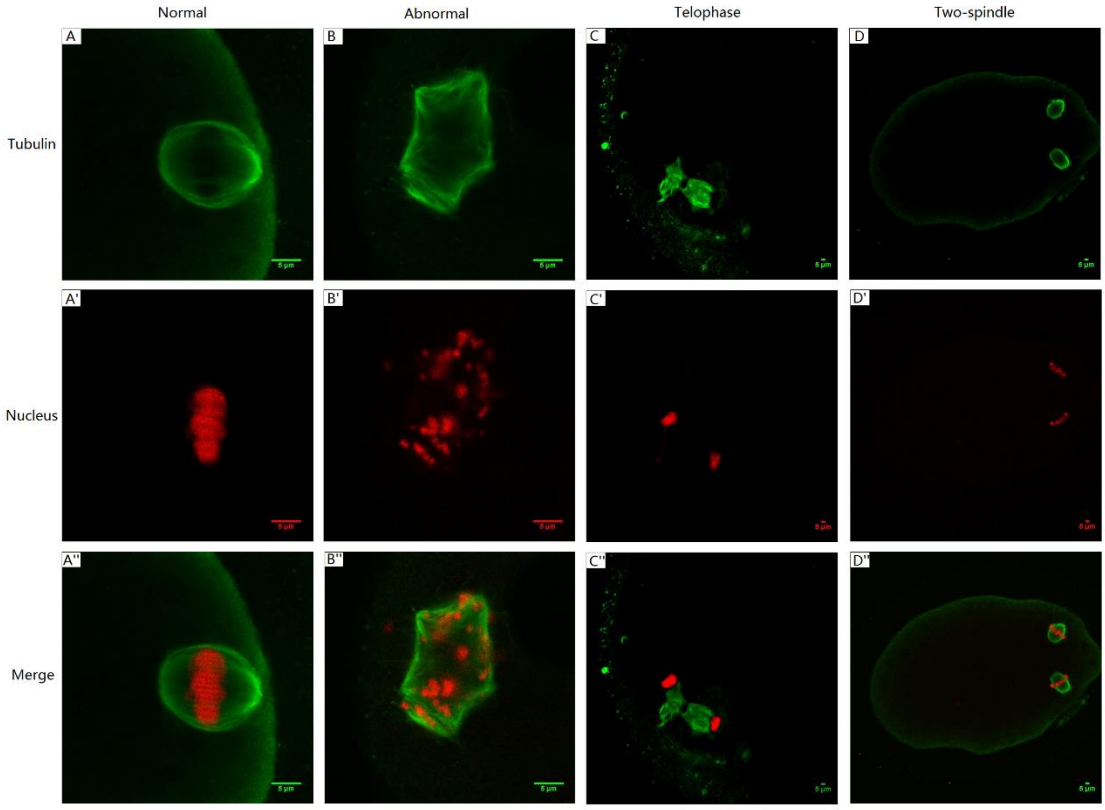
Source of mouse oocytes

GV mouse oocytes were isolated from the ovaries of 8 to 14-week-old female LT/SvEiJ mouse (JAX stock #006252) (n=25) and control B6D2F1 (n=15) mice 44-48h post intraperitoneal injection of 7.5IU/ml PMSG (Folligon®, Intervet) and were cultured in IVM medium for 16h at 37°C in 6% CO_2, 5% O_2. The IVM medium consisted of Minimum Essential Medium Alpha (α-MEM) with GlutaMAX™ (Invitrogen, Life Technologies) supplemented with 5% (v/v) heat-inactivated fetal bovine serum (FBS; Gibco BRL, Life Technologies), 5µg/ml insulin, 5µg/ml transferrin, 5ng/ml selenium (BD Bioscience), 50mIU/ml FSH and 50mIU/ml hCG (Puregon, Organon) (Vanhoutte et al., 2009). In-vivo matured MI and MII mouse oocytes were obtained by priming LT/Sv (n=40) and B6D2F1 (n=20) mice with 7.5IU/ml PMSG, followed by 7.5IU/ml hCG (Chorulon®, Intervet), 48h later. Oocytes were recovered 14h post-hCG. Cumulus cells were removed by short incubation in 200IU/ml hyaluronidase. Potassium simplex–optimized medium (KSOM) was used for culture, while HEPES-buffered KSOM was used for manipulation. Both media were supplemented with 4mg/mL bovine serum albumin (BSA; Calbiochem) and oocytes were cultured at 37°C in 6% CO_2 and 5% O_2 (Lawitts and Biggers, 1991).

Spindle and IP_3R1 staining

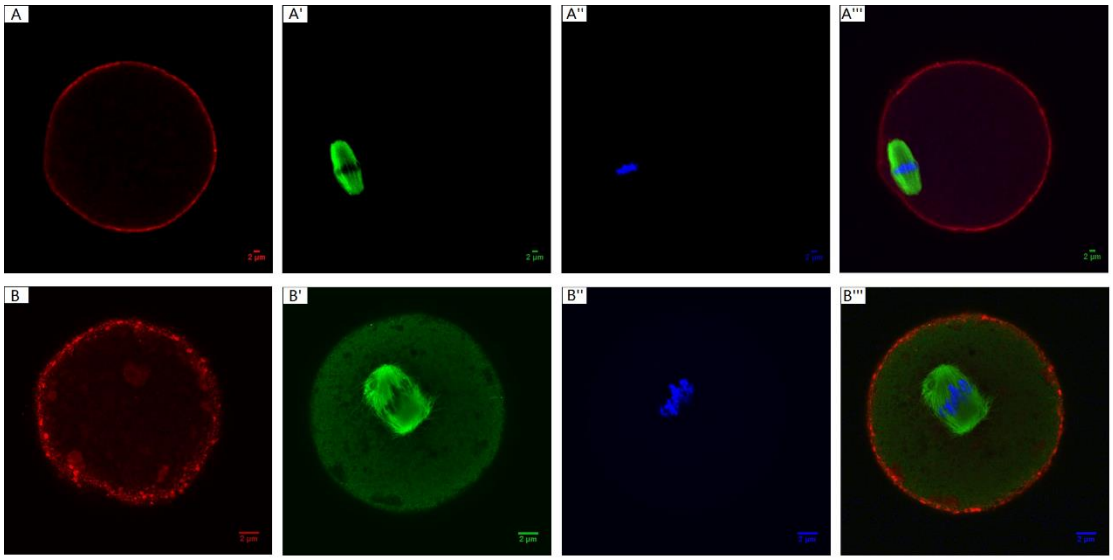
Both human and mouse oocytes were fixed in a microtubule-stabilizing buffer, as previously described (Mattson and Albertini, 1990, Heindryckx et al., 2011). Briefly, stripped in-vitro cultured or in-vivo matured oocytes were simultaneously fixed and extracted in a microtubule-stabilizing buffer (0.1M PIPES, 5mM $MgCl_2$, 2.5mM EGTA, 0.01% aprotinin, 1mM dithiothreitol, 50% deuterium oxide, 1pM taxol, 0.1% Triton X-100, and 3% formalin) for 30min at 37°C. Following 3 times intensive washes (15min), oocytes were subsequently stored at 4°C in PBS-azide until fluorescence staining. At staining, oocytes were incubated overnight at 4°C with a 1/1 mixture of mouse monoclonal anti-α and β-tubulin (1/200) and/or a primary antibody (1/1000) against the IP_3R1 in mouse oocytes (rabbit, polyclonal, KU Leuven) (Parys and Bezprozvanny, 1995). After washing (3x15min), samples were treated with the secondary antibodies Alexa Fluor 488 goat anti-mouse IgG (H+L) (Molecular Probes) (1/200) and/or CY3 donkey anti-rabbit IgG (H+L) (Jackson ImmunoResearch) (1/500) for 1h, followed by extended washing (3x15min). Meanwhile, the negative controls were treated without primary antibody but with the secondary antibodies alone. In addition, chromosomes were stained with Ethidium Homodimer-2 for one hour for human and 20µg/mL Hoechst-33258 for 30min for mouse oocytes, respectively. Finally, the oocytes were mounted in Mowiol containing 0.01% phenylenediamine and imaged using a laser scanning confocal microscope, Nikon A1R confocal microscope (Nikon Instruments) with a 60× Plan Apo VC oil immersion objective. The microtubule structure, chromosomes and/or IP_3R1 distribution were obtained from Z-stacks (0.5–0.75µm/Z-step), using ImageJ software. Microtubule configurations and chromosome alignments of mouse oocytes were classified from A to D, as previously described (Combelles et al., 2011). In human oocytes, the spindles were classified as normal (bipolar) (Figure 1 A), or abnormal (mono- or multi-polar) (Figure 1 B). The chromosomes were classified as aligned (Figure 1 A'), or misaligned (>3 chromosomes away from the spindle equatorial region or dispersed with all chromosomes located throughout the spindle) (Figure 1 B') (Combelles et al., 2011; Trapphoff et al., 2016). In human oocytes, telophase I spindle (Figure 1C) and chromosomes (Figure 1C') (Yu et al., 2012), as well as maturation resistant oocytes with two MII spindles (Figure 1D) with aligned chromosomes (Figure 1D') were also observed. The IP_3R1 distribution was classified as uniform (normal) (Figure 2A) or patched (Figure 2B), as suggested previously (Kim et al., 2011).

Figure 1. Classification of spindle structure configuration and chromosomes alignment in human oocytes



(A) Spindle with representative bipolar structure (normal). (B) Spindle with multi-polar irregularities (abnormal). (C) Elongated telophase I spindle perpendicular to the oocyte membrane following 24-hour culture. (D) Maturation resistant oocytes with two bipolar metaphase II spindles. (A') Chromosomes were classified as aligned (normal). (B') Dispersed chromosomes located throughout the spindle (misaligned). (C') Telophase I chromosomes distributed at the ends of the spindle. (D') Two sets of normally aligned chromosomes within a maturation resistant oocyte. Merged images (A''-D''). Scale bars: 5μm.

Figure 2. Classification of IP_3R1 distribution in LT/Sv mouse oocytes



(A) Homogeneous IP_3R1 distribution with clusters on the cortex, forming a complete circle around the oocyte (Uniform). (A') Normal spindle configuration and chromosome alignment (A'') of B4D2F1 in-vivo matured MII oocytes. (B) Heterogeneous non-uniform distribution with patches and disconnection of IP_3R1 (Patched). (B') Un-focused MI spindle poles and not perfectly aligned chromosomes (B'') of LT/Sv mouse in-vivo collected maturation resistant oocyte. (A'''-B''') Merged images of three confocal channels. Scale bars: 2µm.

Intracellular Ca^{2+}-measurements

Human oocytes were loaded with 7.5µM of Ca^{2+}-sensitive dye Fura-2 acetoxymethyl (AM) ester (Invitrogen, Life Technologies) at 37˚C in 6% CO_2, 5% O_2 for 30min and subsequently extensively washed in Cook cleavage medium. Oocytes were placed in glass- bottomed dishes (MatTek Corporation) and Ca^{2+}-imaging was performed on an inverted epi-fluorescence microscope (TH4-200, Olympus Soft Imaging Solutions GmBH) with a 20x objective. Fluorescence was recorded at an emission wave length of ~ 510nm every 5s. Baseline drifting was adjusted before retrieving values for amplitude (value at maximum increase in fluorescence intensity per peak) expressed in arbitrary units (AU). Frequency reflected the total number of oscillations per recording period.

For measuring spontaneous Ca^{2+}-oscillations in mouse oocytes, isolated GV oocytes were loaded with Fura-2 in KSOM-HEPES for 15min. Oocytes were transferred to a drop of IVM medium and Ca^{2+}-signals were recorded every 30s for a duration of 4h. The strontium induced Ca^{2+}-oscillations were recorded every 5s for a duration of 2h, immediately after transferring the mouse oocytes to a drop of Ca^{2+}-free KSOM with 10mM strontium chloride, in the glass-bottomed dish.

Statistical analysis

The Statistical Package for Social Sciences version 21 (SPSS® Statistics, IBM Corp., USA) was used for statistical analysis. Means of multiple groups were compared using ANOVA and Bonferroni's Multiple Comparison Test. Proportions were compared by a contingency table analysis followed by a chi-squared or Fisher's exact test. Differences yielding a *P* value <0.05 were considered as being statistically significant.

Results

Nuclear maturation competence of human oocytes

In the study group, oocytes were analyzed after in-vitro culture, with 34 MI maturation resistant oocytes retrieved at MI stage and 72 IVM oocytes matured from GV (n=13) and MI stage (n=59) (Supplementary Figure 1). The control group consisted of 55 MI maturation resistant oocytes retrieved at GV (n=9) and MI (n=46) stage, and 23 IVM oocytes matured from GV (n=8) and MI (n=15) stage. Moreover, a total of 21 in-vivo matured MII oocytes (including 7 MII oocytes with SERs) were also included as Controls (Supplementary Figure 1).

Spindle morphology was analyzed by tubulin immunofluorescence (Figure 1 A-D). Abnormal spindle configuration was observed in 62% and 60% of maturation resistant oocytes from the Study and Control group respectively, and no significant difference was found (Table 1). IVM MII oocytes revealed the presence of spindle aberrations in 50% of the oocytes from the Study group compared with 30% of the oocytes from the Controls, which was not significantly different between the two groups (Table 1). Interestingly, in the Controls, the observed percentage of abnormal spindles was not significantly different between IVM and in-vivo matured oocytes (30% versus 14%); in contrast, a significantly higher percentage of oocytes with spindle abnormalities was found in the Study IVM group compared with the in-vivo matured group (50% versus 14%, *P*<0.01, Table 1).

Table 1. Comparison of the spindle-chromosome configuration in maturation resistant, IVM and in-vivo matured human oocytes from study and control groups

	Study group		Control group		
	Maturation resistant MI	IVM MII	Maturation resistant MI	IVM MII	In-vivo matured MII
Number of oocytes (N)	34	72	55	23	21
Spindle abnormalities N (%)	21 (62)	36 (50)[a]	33 (60)	7 (30)	3 (14)
Misaligned Chromosomes N (%)	24 (71)	45 (63)[a,b]	35 (64)	8 (35)	4 (19)
Telophase I spindle N (%)	0	9 (13)	1 (2)	0	0
Two-spindle oocyte N (%)	4 (12)	0	0	0	0

Chi-squared test and Fisher's exact test; [a]$P<0.01$ when compare with Control in-vivo matured MII oocytes; [b]$P<0.05$ when compared with Control IVM MII oocytes.

The proportion of oocytes with misaligned chromosomes (Figure 1B') was comparable in maturation resistant oocytes from the Study and Control group (71% versus 64%, Table 1). Moreover, a significantly higher percentage of misaligned chromosomes persisted in the IVM MII oocytes from Study group compared with the Control IVM MII and in-vivo matured MII oocytes (63% versus 35% versus 19%, $P<0.05$). However, the rate of misaligned chromosomes of Control IVM MII oocytes was not significantly different from the Control in-vivo matured MII oocytes (Table 1). The percentage of oocytes at telophase I stage as well as oocytes with two sets of MII spindle-chromosomes was higher in Study group, when compared with the Control oocytes, however, this did not reach significance either (Table 1).

Pregnancy outcomes of patients showing an abnormally high number of immature oocytes

In total, 83 patients were included to evaluate the fertilization rates and subsequent treatment outcomes. For the analysis, the patients from the Study and Control group were further divided in two subgroups respectively, patients in which at least one maturation resistant oocyte was present after IVM (Study-maturation resistant patients versus Control-maturation resistant patients) and patients in which IVM induced meiotic progress in all oocytes (Study-IVM patients and Control-IVM patients), based on the presence of maturation resistant oocytes observed following in-vitro culture. No significant difference was observed in the mean of the patients age, the number of oocytes retrieved at oocyte retrieval across all groups (Table 2). Study-maturation resistant patients showed a significantly lower number of collected in-vivo matured MII oocytes, when compared with Control-maturation resistant patients ($P<0.05$, Table 2).

Our analysis revealed that the mean of the fertilization rate and the number of embryos per transferred (fresh/frozen) did not differ across all groups ($P>0.05$, Table 2). Moreover, no significant difference was observed in the median of the number of frozen embryo transfers in current retrieval and the number of subsequent stimulations have been performed ($P>0.05$, Table 2). Moreover, the clinical pregnancy rate from all cycles was significantly lower in Study-maturation resistant patients when compared with Control-maturation resistant patients and Study-IVM patients ($P<0.05$, Table 2). All pregnant patients from Study-maturation resistant group (n=2) suffered miscarriages in her frozen cycle or a subsequent stimulation. However, no significant difference was observed in the miscarriage rate amongst all subgroups either from the current oocyte retrieval or across all cycles ($P>0.05$, Table 2). Within 11 patients allocated to Study-maturation resistant group, 4 of them present with all immature oocytes at retrieval, representing approximately half the number of analyzed maturation resistant oocytes (16 out of 34). Conversely, from the Study-IVM patients, 38.5% achieved a clinical pregnancy and with all of them resulted live offspring following current oocyte retrieval (Table 2). The clinical pregnancy and live birth rate of Study-IVM patients did not differ from the Control-IVM patients following the current stimulation, the cumulative clinical pregnancy and live birth rate of Study-IVM patients were comparable to the Controls as well (Table 2).

Table 2. Embryological and cycle outcome data in the Study and Control patients for the investigated oocyte retrieval.

	Study group		Control group	
	Maturation resistant patients	IVM patients	Maturation resistant patients	IVM patients
Number of patients (N)	11	13	43	11
Age (years) at this cycle start	33.6±6.0	33.9±5.5	32.8±5.3	33.4±4.7
Number of oocytes retrieved	12.1±8.9	15.9±8.8	14.8±9.6	14.2±7.9
Number of MII oocytes	4.2±4.0[a]	7.1±4.8	10.8±7.2	10.5±6.3
Fertilization rate	0.56±0.31	0.63±0.23	0.72±0.22	0.77±0.19
Number of embryos per transfer (fresh cycles)	1.0±0.9	1.2±0.8	1.6±0.7	1.4±0.7
Number of embryos per tran sfer (frozen cycles)	1.2±0.4	1.2±0.4	1.1±0.2	1.1±0.4
Number of frozen embryo transfers in current oocyte retrieval	0 (0-2)	0 (0-2)	0 (0-5)	0 (0-3)
Number of subsequent stimulations	1 (0-3)	1 (0-4)	1 (0-5)	1 (0-2)
Clinical pregnancy (fresh)	0	2/13 (15.4%)	6/43 (14.0%)	1/11 (9.1%)
Clinical pregnancy plus frozen	1/11 (9.1%)	5/13 (38.5%)	14/43 (32.6%)	3/11 (27.3%)
Pregnancy across all cycles	2/11 (18.2%)[a,c]	9/13 (69.2%)	26/43 (60.5%)	7/11 (63.6%)
Miscarriage rate for fresh cycles	0	0	0	0
Miscarriage rate in current oocyte retrieval	0	0	2/18 (11.1%)	0
Miscarriage for all cycles	2/2 (100%)	1/9 (11.1%)	6/26 (23.1%)	0
Live birth rate for fresh cycles	0	2/13 (15.4%)	3/43 (7.0%)	1/11 (9.1%)
Live birth rate in current oocyte retrieval	0	5/13 (38.5%)	10/43 (23.3%)	3/11(27.3%)
Live birth rate for all cycles	0[a-c]	7/13 (53.8%)	20/43 (46.5%)	6/11 (54.5%)

ANOVA or Kruskal-Wallis or Chi-squared test; [a]*P*<0.05, when compared with Control maturation resistant patients; [b]*P*<0.05, when compared with Control IVM patients; [c]*P*<0.05 when compared with Study IVM patients.

Study/Control maturation resistant patients: patients in which at least one maturation resistant oocyte was observed following in-vitro culture of retrieved immature oocytes. Study/Control IVM patients: patients in which in-vitro culture induced meiotic completion in all retrieved immature oocytes. Values presented as mean ± SD, median (range), number or number/total number (%). Decimal data indicate the fertilization rate (%).

In Control-maturation resistant patients, 32.6% achieved a clinical pregnancy, resulting in a live birth rate of 23.3% following current oocyte retrieval. No significant differences were observed when compared with Control-IVM patients (Table 2). Moreover, the overall pregnancy and live birth rates did not differ when additional cycles were included in the analysis (Table 2). Of note, the treatment outcomes of Control in-vivo matured patients (n=5) were not displayed in Table 2, due to the small sample size. Briefly, the fresh cycles from two patients who presented all oocytes with SERs were cancelled and no subsequent stimulation has been treated. Other two patients had a fresh embryo transfer and a subsequent stimulation, no pregnancy has been achieved. The patient who donated normal in-vivo matured MII oocytes in the investigated oocyte retrieval achieved an ongoing pregnancy in her second cycle.

Defective IP_3R1 distribution and Ca^{2+}-oscillations during maturation and activation in LT/Sv maturation resistant oocytes

Based on the immunostaining pattern of spindle integrity (Figure 2A'-B'), chromosome configurations (Figure 2A''-B'') and IP_3R1 distribution (Figure 2A-B), we found a significantly higher percentage of LT/Sv IVM MII oocytes with both abnormal spindles and misaligned chromosomes, compared with LT/SV in-vivo matured MII oocytes and IVM MII oocytes from the B6D2F1 control (P<0.01, Table 3). Moreover, we observed a higher percentage of patched IP_3R1 distribution in both IVM and in-vivo matured LT/Sv MII oocytes, when compared with in-vivo matured B6D2F1 MII controls (P<0.05, Table 3).

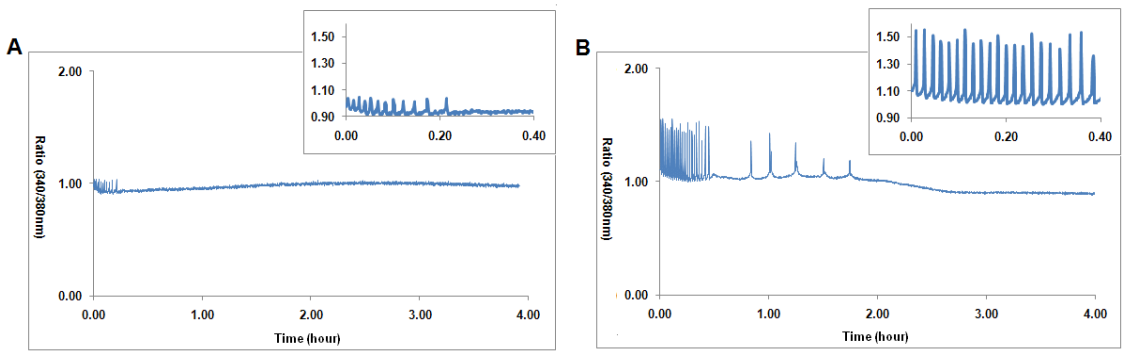
Table 3. Comparison of abnormal spindles, misaligned chromosomes and patched IP_3R1 distribution of LT/Sv maturation resistant MI, IVM MII and in-vivo matured MII oocytes with B6D2F1 controls

	LT/Sv oocytes			B6D2F1 oocytes		
	In-vitro cultured maturation resistant MI	In-vivo collected maturation resistant MI	IVM MII	In-vivo matured MII	IVM MII	In-vivo matured MII
Number of oocytes (N)	19	18	17	18	19	22
Spindle abnormalities N (%)	12 (63)	6 (33)	9 (53)[a-c]	2 (11)	1 (5)	0
Misaligned Chromosomes N (%)	12 (63)	11 (61)	13 (76)[a-c]	2 (11)	3 (16)	3 (14)
Patched IP_3R1 distribution N (%)	4 (21)	4 (22)	8 (47)[b]	4 (22)[b]	4 (21)[b]	0

Chi-squared test and Fisher's exact test; [a]P<0.01 compared with control B6D2F1 IVM MII oocytes; [b]P<0.05 compared with control B6D2F1 in-vivo matured MII oocytes; [c]P<0.05 compared with LT/Sv in-vivo matured MII oocytes.

The maturation competence and the Ca^{2+}-oscillatory pattern of LT/Sv GV oocytes was investigated at GVBD, during in-vitro maturation. After 2h of in-vitro culture, a significantly lower number of LT/Sv GV oocytes (49%, 24/49) transit to the GVBD stage compared with control B6D2F1 (80%, 20/25) GV oocytes (*P*<0.01, data not shown). When Ca^{2+} analysis was performed in oocytes collected at GV stage, only 33% (8/24) of LT/Sv oocytes showed Ca^{2+}-oscillations compared with the majority of control B6D2F1 GV oocytes (75%, 15/20), with a significantly lower amplitude (0.07-0.1 AU versus 0.3-0.7 AU) (*P*<0.01, Figure 3).

Figure 3. Spontaneous Ca^{2+}-oscillations during GVBD of LT/Sv and B6D2F1 oocytes



(A) Ca^{2+}-oscillations during GVBD of LT/Sv oocytes. High frequency of Ca^{2+}-oscillations with a reduced amplitude of 0.3-0.7 in 1-3min intervals during the first hour of measurement. (B) Ca^{2+}-oscillations during GVBD of B6D2F1 oocytes. High frequency Ca^{2+}-oscillations repeated with amplitude of 0.07-0.1 at 20-45s interval, during the first hour of measurement.

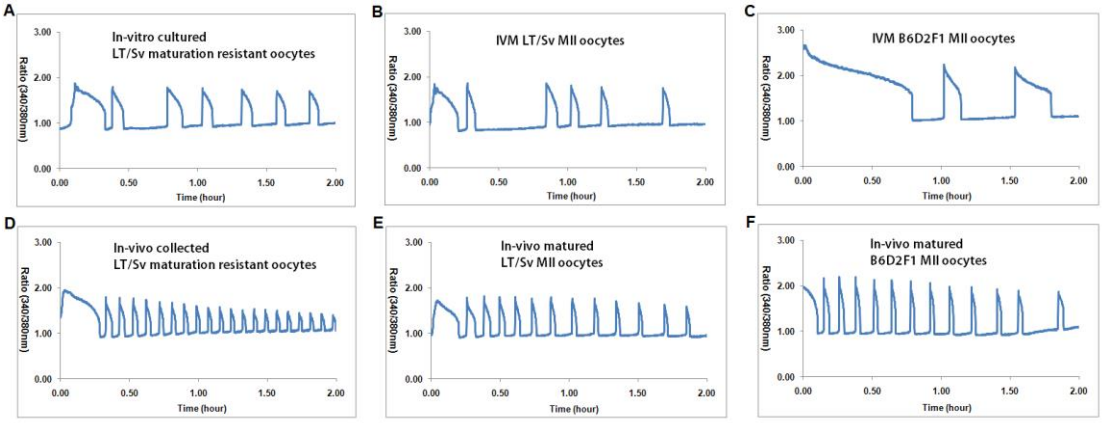
Following the exposure to strontium, the frequency of Ca^{2+}-rise was significantly lower in LT/Sv in-vitro cultured maturation resistant compared with the in-vivo collected LT/Sv maturation resistant and in LT/Sv IVM MII oocytes compared with LT/Sv in-vivo matured MII oocytes (both *P*<0.01, Table 4). Furthermore, LT/Sv in-vitro cultured maturation resistant and IVM MII oocytes showed a higher frequency of Ca^{2+}-oscillations, when compared with IVM B6D2F1 MII oocytes (*P*<0.01, Figure 4A-C, Table 4). Additionally, in-vivo collected LT/Sv maturation resistant oocytes exhibited a significantly higher number of Ca^{2+}-oscillations, compared with in-vivo matured LT/Sv MII and in-vivo matured B6D2F1 MII oocytes (*P*<0.05, Figure 4D-F, Table 4).

Table 4. Ca^{2+}-oscillations in LT/Sv and control B6D2F1 oocytes during parthenogenetic activation with strontium.

	LT/Sv oocytes			B6D2F1 oocytes		
	In-vitro cultured maturation resistant MI	In-vivo collected maturation resistant MI	IVM MII	In-vivo matured MII	IVM MII	In-vivo matured MII
Number of oocytes (N)	23	19	24	25	49	45
Alive oocytes following exposure N (%)	7 (30)[a]	18 (95)	24 (100)[c]	25 (100)	21 (43)[d]	45 (100)
Oocytes showing oscillations N (%)	6 (86)	18 (100)	22 (92)	24 (96)	18 (86)	44 (98)
Frequency of Ca^{2+}-spikes	5.50±3.73[a,c]	14.28±5.83[b,d]	4.59±2.34[b-d]	11.25±4.23[c]	1.50±0.86[d]	11.34±4.96

Chi-squared or ANOVA test; [a]*P*<0.01when compared with LT/Sv in-vivo collected maturation resistant MI oocytes; [b]*P*<0.05 when compared with LT/Sv in-vivo matured MII oocytes; [c]*P*<0.01 when compared with control B6D2F1 IVM MII oocytes; [d]*P*<0.05 when compared with B6D2F1 in-vivo matured MII oocytes.

Figure 4. The strontium induced Ca^{2+}-release pattern in LT/Sv in-vitro cultured maturation resistant, IVM MII and B6D2F1 IVM MII control oocytes (A-C). The Ca^{2+}-release trace of LT/Sv in-vivo collected maturation resistant, in-vivo matured MII oocytes and B6D2F1 in-vivo matured MII oocytes (D-F).



Discussion

Although complete failure of oocytes to complete meiosis maturation during assisted conception cycles is rare, occasional and repetitive maturation resistant does occur in infertile patients (Bar-Ami et al., 1994, Avrech et al., 1997, Beall et al., 2010, Heindryckx et al., 2011). Patients presenting with an abnormally high number of immature oocytes at retrieval show a great possibility of exhibiting maturation resistant oocytes, however, the influence of such event on the nuclear maturation competence, as well as on the subsequent pregnancy outcomes of these patients have not been investigated. In this study, we reveal that patients presenting with a high proportion of immature oocytes retrieved at oocyte retrieval (>40%), in addition to one or more maturation resistant oocytes following routine ovarian stimulation, exhibited abnormal nuclear maturation and failed to achieve a live birth. The treatment and clinical utilization of IVM oocytes of these patients in IVF transfers should be reassessed. Similar nuclear abnormalities were observed in IVM and maturation resistant oocytes from a maturation resistant mouse model, in addition to aberrant Ca^{2+}-signaling observed during oocyte maturation and following artificial activation.

Previous reports have indicated that in ovarian stimulation cycles with a high percentage of maturation resistant oocytes present after oocyte retrieval, fertilization rates and blastomere numbers per embryo are significantly reduced (Bar-Ami et al., 1994). From our data, patients with >40% immature oocytes, in which at least one maturation resistant oocyte was observed after in-vitro culture (Study-maturation resistant patients), did not show reduced fertilization rates, compared with control patients. However, no live birth was achieved in this group of patients. In support of this poor clinical outcome, we observed that the IVM oocytes from the Study group had a significantly higher proportion of spindle abnormalities compared with the oocytes in the control in-vivo matured group and a significantly higher proportion of chromosome abnormalities compared with IVM and in-vivo matured MII oocytes from Controls. Although some reports suggest IVM treatment as a suitable strategy for patients showing a high number of immature oocytes (Reichman et al., 2010), our results indicate that this approach should be considered with caution, as it may not be applicable for patients with more than 40% immature oocytes. Moreover, a high percentage of spindle-chromosome abnormalities was observed in maturation resistant oocytes from both Study and Control group.

Correct chromosome segregation during oocyte meiotic divisions is a crucial factor for successful embryogenesis. Defects during this process lead to reduced developmental outcomes and are associated with subsequent pregnancy loss (Maciejewska et al., 2009). Moreover, SAC malfunctions, as well as dysregulation of maturation promoting factor (cyclin B) activity have been linked to reduced pre-implantation development and implantation potential (Ciemerych and Kubiak, 1998, Maciejewska et al., 2009, Hoffmann et al., 2012). The observed

defective spindle-chromosome configurations of oocytes from the Study group, may lead to the over-activation of SAC as previously reported (Maciejewska et al., 2009). As such, spindle configuration and SAC activation of in-vivo matured MII oocytes from the Study group requires further validation.

Moreover, we utilized our maturation resistant LT/Sv mouse model to investigate Ca^{2+}-related cytoplasmic maturation markers, in detail, to provide further clues regarding the origin of maturation resistance in human oocytes. The LT/Sv mice showed a high percentage of maturation resistant MI oocytes from both in-vivo and in-vitro collection as observed in human Study-maturation resistant patients, moreover, LT/Sv IVM oocytes displayed aberrant spindle-chromosomes configurations, similar to observed human IVM oocytes from the Study group. We attempted to analyze IP_3R1 distribution and ionomycin induced Ca^{2+}-release patterns (data not shown) in human maturation resistant oocytes from the Study and Control groups. However, due to the limited number of maturation resistant oocytes collected, we could not draw any relevant conclusions. The impaired Ca^{2+}-signaling during IVM observed in this study, might explain the observed high MI arrest rate of LT/Sv oocytes following IVM as reported (O'Neill and Kaufman, 1987). Deregulated Ca^{2+}-signaling could be attributed to altered oocyte metabolism (Lam and Galione, 2013, Williams et al., 2013) and impaired IP_3R1 distribution (Lefevre et al., 1997). However, following strontium exposure, we also found higher frequencies of Ca^{2+}-rises in both in-vitro cultured and in-vivo collected LT/Sv maturation resistant oocytes, when compared with B6D2F1 IVM and in-vivo matured MII oocytes, respectively. Results from our Ca^{2+} analysis of LT/Sv maturation resistant oocytes suggest a method to investigate that cytoplasmic competence of human IVM oocytes from the Study group.

Several studies have demonstrated that GV nuclear or cytoplasmic transfer represent potential treatment techniques to alleviate oocyte meiosis arrest, shown in the maturation resistant mouse model (Hoffmann et al., 2012) and for human oocyte maturation defects (Cohen et al., 1997, Zhang et al., 1999). Spindle transfer has also been successfully applied for the treatment of mitochondrial disease (Neupane et al., 2014). However, considering the compromised IP_3R1 distribution and impaired spindle-chromosome configuration observed in both human and mouse IVM and MR oocytes from our Study groups, the application of these methodologies requires further validation. Certainly, in depth embryo developmental and safety studies are required prior to the clinical implementation of the treatments.

In conclusion, patients with a high proportion of immature oocytes retrieved at oocyte retrieval (>40%), in addition to one or more maturation resistant oocytes following routine ovarian stimulation, exhibited abnormal nuclear maturation and failed to achieve subsequent pregnancy. The treatment and further application of IVM oocytes from these patients should be

reassessed. Defective Ca^{2+}-signaling at GVBD of in-vitro cultured LT/Sv maturation resistant oocytes and abnormal Ca^{2+}-signaling of in-vivo collected LT/Sv maturation resistant oocytes following strontium exposure, point to broader impaired cytoplasm and oocyte quality. Our findings, demand further research into human maturation resistant oocytes, particularly in patients with a high number of immature oocytes.

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Conflict of interest

The authors declare no conflict of interest.

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Chapter 4

4. Culture conditions affect Ca^{2+} signaling in artificially activated mouse and human oocytes

Objective

Ionomycin is a Ca^{2+}-selective ionophore commonly used for assisted oocyte activation (AOA), to overcome failed fertilization. In the second study of this thesis, we investigated the effect of external ionomycin and Ca^{2+} concentrations in culture media during AOA on both activation efficiency and embryonic developmental potential, using both mouse and human oocytes.

Conclusion

The Ca^{2+} signaling and subsequent embryo developmental potential of mouse oocytes is influenced by the concentration of both ionomycin and external Ca^{2+} supplemented in the culture media. In human oocytes, the rise in Ca^{2+} levels provoked by 10μM ionomycin, was affected by the total calcium concentration present in the commercial media. The influence of commercial media on the amount of Ca^{2+} rise and subsequent treatment outcomes should be considered when applying AOA in the clinic.

Culture conditions affect Ca^{2+} rise in artificially activated mouse and human oocytes

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ABSTRACT

Inconsistent fertilization and pregnancy rates have been reported by different laboratories after application of ionomycin as a clinical method of assisted oocyte activation (AOA) to overcome fertilization failure. Using both mouse and human oocytes, we investigated the effect of ionomycin and Ca^{2+} concentrations on the Ca^{2+}-oscillatory pattern and embryonic developmental potential. In mouse, we found that the application of 5µM ionomycin in KSOM or 10µM ionomycin in Ca^{2+}-free KSOM medium, significantly reduced the amount of Ca^{2+} flux and resulted in blastocyst formation failure compared to 10µM ionomycin in KSOM medium. Increasing Ca^{2+} concentration up to 3 or 6 times did not benefit mouse embryonic developmental potential. Similarly, the Ca^{2+} rise provoked by 10µM ionomycin in human oocytes increased along with the total calcium concentration present in the commercial media. Remarkably, we observed significantly reduced mouse embryo development when performing AOA during 10 minutes in SAGE and Vitrolife medium in contrast to COOK medium, with the same culture system supported from post activation to blastocyst formation stage in different AOA groups. In conclusion, both the concentration of ionomycin and Ca^{2+} supplemented in culture media applied during AOA can have a significant impact on the Ca^{2+} rise and further embryonic developmental potential.

KEYWORDS: Ionomycin, Ca^{2+} signaling, assisted oocyte activation, fertilization failure

Introduction

Intracytoplasmic sperm injection (ICSI) is currently the most effective and widely applied technique for treating male-factor infertility. It is also routinely used for indications of failed or low fertilization after treatment with conventional in vitro fertilization (IVF). Research shows that fertilization rates after ICSI are between 70% to 80%, which is considerably higher than other assisted procedures (Palermo *et al.* 2009; Neri *et al.* 2014). However, complete fertilization failure still occurs in approximately 1-5% of all ICSI cycles and can reoccur in subsequent treatments (Flaherty *et al.* 1998; Esfandiari *et al.* 2005). The principal cause of ICSI failure is generally associated with a deficiency in the oocyte activation process (Rawe *et al.* 2000; Yanagida 2004; Swain and Pool 2008; Neri *et al.* 2014). Oocyte activation is triggered by the sperm factor phospholipase C zeta, which mediates specific oscillations in Ca^{2+} concentrations within the ooplasm (Saunders *et al.* 2002; Tesarik 2002). Further downstream, binding of inositol 1,4,5-trisphosphate to its receptors (IP_3Rs) in the oocyte generates the Ca^{2+} release required for successful activation, fertilization and subsequent embryonic development (Ramadan *et al.* 2012; Wakai *et al.* 2013). As such, both sperm and oocyte factors play an equally imperative role during the oocyte activation process. Irregularities in the characteristic Ca^{2+} oscillation pattern in the oocyte during activation may thus prevent fertilization and reduce embryonic developmental potential (Ajduk *et al.* 2011).

For couples experiencing failed fertilization after ICSI, heterologous ICSI models, such as the mouse oocyte activation test and the mouse oocyte Ca^{2+} analysis have been successfully used to establish activation deficiencies and determine whether they are sperm or oocyte-related (Rybouchkin *et al.* 1996; Araki *et al.* 2004; Heindryckx *et al.* 2005). Following diagnosis, the technologies named assisted oocyte activation (AOA) are commonly applied to overcome fertilization failure. Thus far, a number of physical, mechanical and/or chemical AOA methods have been evaluated to achieve activation, including electrical pulses, modified ICSI procedures, as well as Ca^{2+} ionophores (Tesarik *et al.* 2002; Egashira *et al.* 2009; Nasr-Esfahani *et al.* 2010; Vanden Meerschaut *et al.* 2014). The most widely utilized artificial activating agents for human oocytes are Ca^{2+} selective ionophores, ionomycin and calcimycin (also known as A23187) (Heindryckx *et al.* 2008; Ebner *et al.* 2012). In contrast to strontium that induces a series of Ca^{2+} oscillations as elicited by sperm penetration during fertilization, the Ca^{2+} ionophore triggers a single Ca^{2+} transient in mouse and human oocytes (Vanden Meerschaut *et al.* 2014; Nikiforaki *et al.* 2016; Tosti and Ménézo 2016). Remarkably, neither Ca^{2+} rises nor oocyte activation is observed in our group when applying strontium as an AOA method in human (unpublished data).

The availability and application of a variety of AOA technologies across laboratories has led to inconsistent results, with discordant fertilization and pregnancy data reported to date (Ebner *et al.* 2012; Montag *et al.* 2012). Moreover, some studies describe similar outcomes with AOA

compared to routine ICSI, with no significant improvement in fertilization rates (Borges *et al.* 2009b; Borges *et al.* 2009a). Although such discrepancies may be attributed to a number of patient related factors, such as fertility background, the lack of diagnostic tests to accurately determine activation deficiencies, the variability within the AOA protocols themselves may also play a role (Vanden Meerschaut *et al.* 2014). It was recently demonstrated that the commercial available calcimycin was less efficient in inducing oocyte activation when compared to ionomycin (Nikiforaki *et al.* 2016). Furthermore, some groups describe the concomitant injection of Ca^{2+} chloride during ICSI in their AOA protocol (Heindryckx *et al.* 2008; Vanden Meerschaut *et al.* 2012), while others limit the AOA protocol to a one-time ionophore exposure. Such variations make standardization difficult. Moreover, the medium used for embryo culture following activation may affect further embryonic development (Malik *et al.* 2014). The overall compositions vary notably in the human embryo culture media (Morbeck *et al.* 2014a; Morbeck *et al.* 2017). However, the effects of specific culture components, such as the concentration of Ca^{2+} in the medium ($[Ca^{2+}]$) applied in clinical AOA has not been thoroughly considered. Studies using bovine and porcine oocytes reveal that AOA using ionomycin combined with a specific culture medium containing a certain amount of Ca^{2+}, increases activation efficiency and significantly improves embryonic development (Miyoshi *et al.* 2008; Fernandes *et al.* 2013; Malik *et al.* 2014).

Nevertheless, the exact mechanism of ionomycin induced Ca^{2+} rise within mammalian oocytes remains unknown. To activate cells, ionomycin induces Ca^{2+} influx from the surrounding culture medium by transporting ions across membranes to equilibrate concentrations, or acts directly on intracellular organelles, as observed in starfish oocytes and zygotes (Mason and Grinstein 1993; Morgan and Jacob 1994; Vasilev *et al.* 2012). The impact of certain parameters on the efficiency of ionomycin, such as the induced Ca^{2+}-oscillatory pattern during oocyte activation, remains to be investigated. Furthermore, specific effects of varying Ca^{2+} media concentrations on subsequent outcomes such as blastocyst formation rate are still unknown. In this study, we establish the intra-oocyte Ca^{2+} response to different concentrations of ionomycin and the effect of $[Ca^{2+}]$ in the surrounding culture medium. Primarily, we investigated the effects of the ionomycin and $[Ca^{2+}]$ concentrations as an AOA method on mouse oocyte activation, and three commercial available culture media were further evaluated with mouse oocytes. We hypothesized that the external ionomycin concentration and the $[Ca^{2+}]$ in the culture medium could affect AOA efficiency and impact subsequent embryonic developmental potential in both mouse and human. To this end, we further considered the effect of the surrounding $[Ca^{2+}]$ from commercial media on the Ca^{2+}-oscillatory pattern of human oocytes.

Materials and methods

All chemicals were purchased from Sigma-Aldrich 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). All procedures involving handling and sacrificing the animals were approved by the Ghent University Hospital Ethical Committee for Laboratory Animals (ECD no. 14/45). Written informed consent were obtained from all patients.

Source and culture of mouse oocytes

6-10-week-old B6D2F1 hybrid female mice were stimulated with 7.5IU pregnant mare serum gonadotrophin (PMSG, Folligon®, Intervet, Boxmeer, The Netherlands), followed by 7.5IU human chorionic gonadotrophin (hCG, Chorulon®, Intervet, Boxmeer, The Netherlands), 46-48 hours later. Metaphase II (MII) oocytes were harvested 12-14 hours following hCG injection in HEPES buffered potassium simplex optimized medium (KSOM-HEPES) supplemented with 4mg/ml bovine serum albumin (BSA, Calbiochem). Cumulus cells surrounding the oocytes were removed by treatment with 200 IU/ml hyaluronidase 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 (1.71mM [Ca^{2+}]).

Source and culture of human oocytes

Human spare oocytes were donated by patients (22-43 years old) undergoing ICSI/IVF treatment at the Ghent University Hospital between Aug 2016 and Dec 2016. Women undergoing a hormone stimulated cycle were administrated with a GnRH agonist (Decapeptyl; Ferring) or antagonist (Cetrotide;Merck Serono). Ovarian stimulation was performed with administration of hMG (Menopur; Ferring) or recombinant FSH (Gonal-F; Merck Serono) at a dose of 112.5–300IU daily and ovulation was induced with 5000IU hCG (Pregnyl;MSD). Oocytes were enzymatically denuded by brief exposure to 80IU/ml hyaluronidase (Irvine Scientific), followed by mechanical denudation before ICSI. The nuclear status was assessed and classified as GV (presence of a Germinal Vesicle structure), Metaphase I (MI) (absence of both a polar body (PB) and a GV structure) or MII stage (presence of a PB and absence of a GV structure). Donated GV oocytes were further cultured in medium 199 supplemented with 10ng/ml epidermal growth factor, 1mg/ml estradiol, 10mIU/ml recFSH, 0.5mIU/ml hCG, 1mM l-glutamine, 0.3mM sodium pyruvate, 0.8% (v/v) human serum albumin (Red Cross, Belgium), 100IU/ml penicillin G and 100 mg/ml streptomycin sulfate at 37°C under 6% CO_2, 5% O_2 for 24 hours. Immature MI stage oocytes were cultured in Sydney IVF COOK cleavage medium (CC) (Cook Ireland Ltd) for 3 hours or 24 hours based on 1st PB extrusion.

Exposure of mouse and human oocytes to combinations of ionomycin and culture medium

In the first set of experiments, mouse MII oocytes were exposed to 5μM, 10μM or 15μM ionomycin (cat. no. I9657, Sigma-Aldrich, Bornem, Belgium) dissolved in 1x[Ca^{2+}] KSOM for 10 minutes, respectively. Ionomycin was made as 5mM, 10mM and 15mM stock solution in dimethyl sulfoxide (DMSO) to ensure the amount of DMSO used was comparable across the different groups. Furthermore, mouse MII oocytes were exposed to 10μM ionomycin dissolved in nominally [Ca^{2+}]-free KSOM (without added Ca^{2+}, but with ~10 μM), 1x[Ca^{2+}] KSOM, 3x[Ca^{2+}] KSOM or 6x[Ca^{2+}] KSOM for 10 minutes respectively. [Ca^{2+}]-free KSOM, 1x[Ca^{2+}] KSOM, 3x[Ca^{2+}] KSOM and 6x[Ca^{2+}] KSOM were prepared by adding 0mM, 1.71mM, 5.13mM, and 10.26mM $CaCl_2$ in home-made [Ca^{2+}]-free KSOM. The osmotic concentrations of culture media were comparable in the different [Ca^{2+}] KSOM groups. In the third group of experiments, mouse MII oocytes were exposed to 10μM ionomycin dissolved in one of the three commercial IVF media (as outlined below in human) for 10 minutes. Further culture was performed in the same culture media.

All parthenogenetically activated mouse oocytes were cultured in KSOM with 2μg/ml Cytochalasin D (CCD) for 4 hours after ionomycin exposure. CCD is used to prevent extrusion of the second PB to create diploid parthenogenetic embryos. Mouse MII oocytes activated with 10mM strontium chloride dissolved in [Ca^{2+}]-free KSOM with 2μg/ml CCD for 4 hours were used as positive control. After activation, the mouse MII oocytes were extensively washed and cultured in 1xKSOM for the first 60-72 hours post-activation. Subsequently, embryos were transferred to Sydney IVF COOK blastocyst medium (Cook Ireland Ltd). Embryo development was assessed at 24 hours (two-cell), 72 hours (morula/early blastocyst) and 96 hours (blastocyst) post-activation. Three replicates were performed for each concentration combination. A minimum of 18 oocytes were included in each group.

In human, *in vitro* matured GV-MII 24 hours, MI-MII 3 hours and MI-MII 24 hours oocytes were exposed to 10μM ionomycin dissolved in one of the following commercial IVF media for 10 minutes: CC medium, Quinns Advantage® Fertilization medium (SAGE) (Cooper Surgical Inc, CT, USA), Vitrolife IVF™ (Vitrolife, Göteborg, Sweden) and Sydney IVF COOK embryo biopsy [Ca^{2+}]-free medium (Cook Ireland Ltd, Ireland).

Ca^{2+} imaging in mouse and human oocytes following ionomycin exposure

Mouse and human MII oocytes were loaded with 7.5μM of the ratio metric Ca^{2+} sensitive dye Fura-2 acetoxymethyl (AM) ester (Invitrogen, Life Technologies Europe B.V., Belgium) at 37˚C, 6% CO_2 and 5% O_2 for 30 minutes and then washed extensively. Subsequently, oocytes were placed in 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 20x objective. Fluorescence was recorded at an emission wave length of ~ 510 nm every 5 seconds for 10 minutes. The baseline was set first, for one minute prior to the addition of ionomycin. The ratio of both Ca^{2+} induced signals (340nm/380nm) was proportional to the concentration of free intracellular Ca^{2+} (expressed in arbitrary units, AU). During acquisition, fluorescently loaded oocytes were exposed to different concentrations of ionomycin and/or incubated with culture media containing different [Ca^{2+}].

All oocytes were distributed randomly across the different groups and tested within 2 hours after assessing the maturation state. A maximum of 3 oocytes were measured simultaneously. Baseline drifting was adjusted before retrieving values for amplitude (value at maximum increase in fluorescence intensity per peak) expressed in arbitrary units (AU). Ca^{2+} rises were compared for time to peak (time in minutes from baseline to value at maximum), frequency (the total number of oscillations per recording period) and area under the curve (AUC) of the Ca^{2+} rise (calculated and expressed in AU x minutes).

The total calcium concentrations of three commercial media and KSOM were analyzed by the polarized Energy Dispersive X-Ray Fluorescence spectrometer (EDXRF) system (Rigaku NEX CG). The accuracy of this technology is 0.001 molar (40 mg/L for calcium), while the detection limit is around 0.0001 molar (Silva *et al.* 2012).

Statistical analysis

The Statistical Package for the Social Sciences version 21 (SPSS® Statistics, IBM corp., NY, USA) was used for statistical analysis. Means (time to peak, RA, AUC and calcium concentration) of the two groups were compared by Student's t-test, while multiple groups were compared using one-way ANOVA and Bonferroni's Multiple Comparison Test. Proportions (activation and blastocyst formation rate) were compared by a contingency table analysis followed by a chi-square or Fisher's exact test. Differences yielding a P value < 0.05 were considered as being statistically significant.

Results

Different ionomycin concentrations elicited distinct Ca^{2+} responses in mouse oocytes

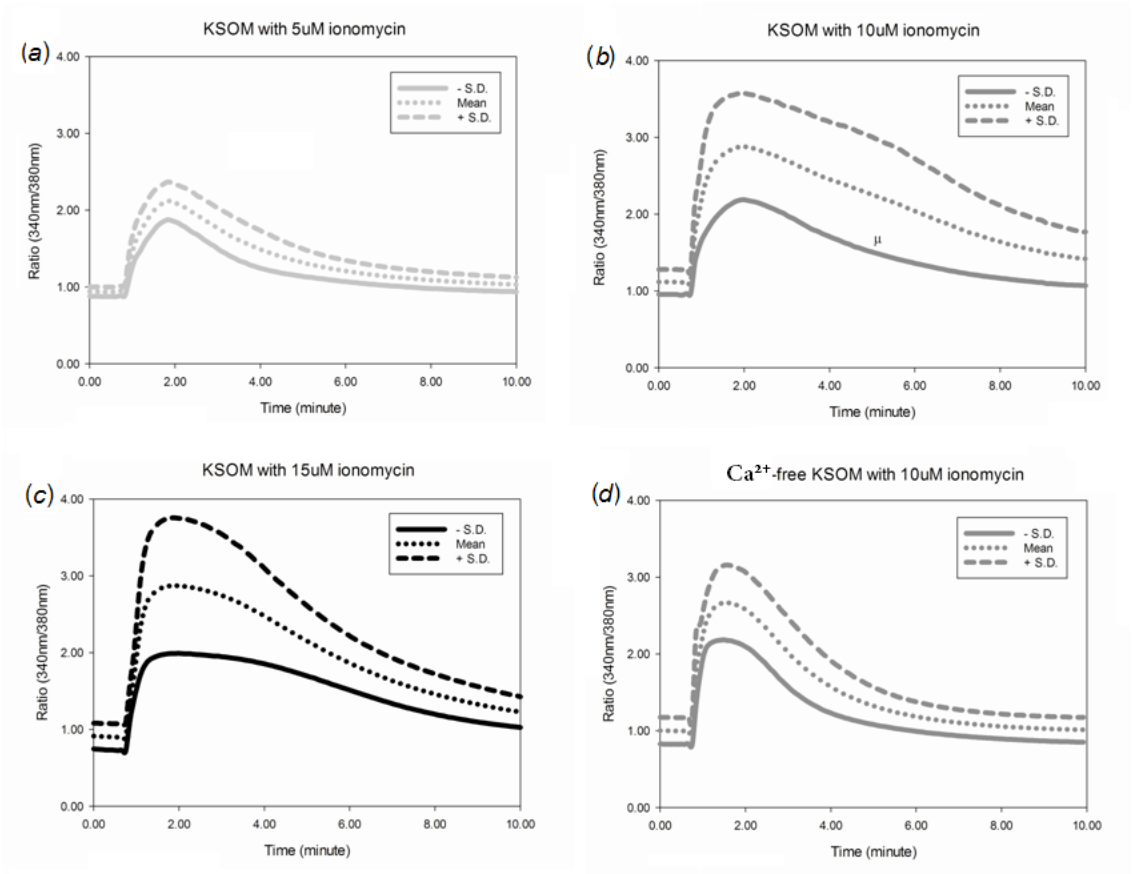
Following the exposure of mouse MII oocytes to ionomycin, the intracellular Ca^{2+} level promptly increased. The time to peak (1.22 ± 0.39 min versus 1.27 ± 0.41 min versus 1.25 ± 0.43 min) did not differ among the three groups with different concentrations of ionomycin. The RA of the Ca^{2+} rises and the AUC were significantly higher in the mouse oocytes exposed to 10μM ionomycin compared to 5μM ionomycin (Table 1, Fig. 1*a*, *b*). However, when the concentration of ionomycin increased to 15μM, there was no significant increase of the RA and the AUC of the Ca^{2+} rise compared to 10μM group (Table 1, Fig. 1*b*, *c*).

Table 1. The impact of the ionomycin concentrations on the Ca^{2+} rise of mouse oocytes cultured in 1x[Ca^{2+}] KSOM

	No. oocytes	RA of the Ca^{2+} rise	AUC of the Ca^{2+} rise
5μM ionomycin	20	1.18 ± 0.25	4.20 ± 1.47
10μM ionomycin	20	1.71 ± 0.60[a]	8.82 ± 4.19[b]
15μM ionomycin	19	1.98 ± 0.82[a]	10.93 ± 3.84[b]

Data represent the mean ± s.d. [a]$P < 0.05$ when compared to RA of 5μM ionomycin group, [b]$P < 0.01$ when compared to AUC of 5μM ionomycin group. RA, relative amplitude. AUC, area under the curve.

Fig. 1. Ca^{2+} response in mouse oocytes following ionomycin exposure.



The single Ca^{2+} rise was characterized by a rapid upstroke followed by a slower decline towards the baseline. Mean ± S.D.

Extracellular Ca^{2+} amount influenced intracellular Ca^{2+} responses in mouse oocytes upon application of 10μM ionomycin as AOA

We assessed the impact of different [Ca^{2+}] in the surrounding media on the 10μM ionomycin induced intracellular Ca^{2+} level of mouse oocytes. In the first set of experiments, we examined the effect of different [Ca^{2+}] in the culture media on the ionomycin induced Ca^{2+} rise. The RA of the Ca^{2+} rise was similar in 1x[Ca^{2+}] KSOM and Ca^{2+}-free KSOM medium, however, the AUC was significantly larger in 1x[Ca^{2+}] KSOM compared to Ca^{2+}-free KSOM medium (8.82 ± 4.19 versus 5.03 ± 2.31 AU, $P < 0.01$) (Table 2, Fig. 1*b*, *d*). In the second set of experiments, we investigated the ionomycin induced intracellular Ca^{2+} level dissolved in KSOM culture media with 3 or 6 times [Ca^{2+}]. The RA and AUC of the ionomycin induced intracellular Ca^{2+} rise was significantly higher in 6x[Ca^{2+}] KSOM when compared to 3x[Ca^{2+}] KSOM, however, no significant difference was found in either RA or AUC of the Ca^{2+} rise when comparing the 3x[Ca^{2+}] KSOM to the 1x[Ca^{2+}] KSOM group (Table 2).

Table 2. The impact of the extracellular Ca^{2+} concentration on the intracellular Ca^{2+} response in mouse oocytes upon application of 10μM ionomycin

	No. oocytes	RA of the Ca^{2+} rise	AUC of the Ca^{2+} rise
Ca^{2+}-free KSOM	21	1.68 ± 0.58[a]	5.03 ± 2.31[b,c]
1x[Ca^{2+}] KSOM	20	1.71 ± 0.60[a]	8.82 ± 4.19
3x[Ca^{2+}] KSOM	16	1.64 ± 0.38[a]	7.87 ± 3.34[b]
6x[Ca^{2+}] KSOM	17	2.35 ± 0.84	12.48 ± 7.15

Data represent the mean ± s.d. [a]$P < 0.05$ when compared to RA of 6x[Ca^{2+}] KSOM medium, [b]$P < 0.05$ when compared to AUC of 6x[Ca^{2+}] KSOM medium, [c]$P < 0.01$ when compared to AUC of 1x[Ca^{2+}] KSOM medium. RA, relative amplitude. AUC, area under the curve.

Three different clinical IVF media differentially affect the pattern of Ca^{2+} rise

We further assessed the influence of different commercially available culture media on the 10μM ionomycin induced intracellular Ca^{2+} level in mouse oocytes. The RA of the Ca^{2+} rise was not significantly different amongst the three groups. However, the AUC of the Ca^{2+} rise provoked in the commercial Vitrolife culture medium was significantly lower compared to the other two media (7.03 ± 2.34 versus 9.45 ± 5.06 versus 10.96 ± 4.87 min x AU, $P < 0.01$) (Table 3).

Table 3. The impact of calcium concentration on the Ca^{2+} rise of mouse oocytes following 10 μM ionomycin exposure, comparison of three different commercially available IVF culture media

	No. oocytes	RA of the Ca^{2+} rise	AUC of the Ca^{2+} rise
CC medium	21	2.01±0.42	9.45±5.06
SAGE medium	17	1.90±0.29	10.96±4.87
Vitrolife medium	19	1.83±0.26	7.03±2.34[a]

Data represent the mean ± s.d. [a]$P < 0.05$ when compared to AUC of SAGE medium with $P < 0.05$. RA, relative amplitude. AUC, area under the curve.

Effect of different concentrations of ionomycin and [Ca^{2+}] on mouse embryonic development

Mouse MII oocytes were activated with different concentrations of ionomycin or 10μM ionomycin dissolved in different culture media, as aforementioned. There was no significant difference in the 2-cell formation rate amongst the 5μM, 10μM and 15μM ionomycin groups. However, all three groups showed significantly lower 2-cell formation rate compared to the strontium control ($P < 0.05$, Table 4). Interestingly, no blastocyst formation was observed in the 5μM ionomycin group; in contrast, a significantly higher blastocyst rate (79%) was obtained when 10μM ionomycin was used ($P < 0.01$), while 42% blastocysts were formed in the 15μM ionomycin group (Table 4). Except for the 10μM ionomycin group, exposure to both 5μM and 15μM ionomycin resulted in a significantly lower number of blastocysts compared to the strontium control ($P < 0.01$, Table 4).

When comparing three different [Ca^{2+}] in the culture medium during the ionomycin exposure, there was no significant difference in 2-cell formation rate within the 1x[Ca^{2+}] KSOM, 3x[Ca^{2+}] KSOM and 6x[Ca^{2+}] KSOM group (Table 4). However, we observed a significantly decreased activation rate in all three ionomycin groups, when compared to the strontium control ($P < 0.05$, Table 4). Moreover, a significantly decreased blastocyst formation rate was observed in 6x[Ca^{2+}] KSOM group compared to the strontium control ($P < 0.05$, Table 4). Interestingly, we found a significantly lower 2-cell formation rate in Ca^{2+}-free KSOM, compared to the other three KSOM groups ($P < 0.05$), and no blastocysts were obtained in the Ca^{2+}-free KSOM group (Table 4).

We further investigated the embryonic development potential after ionomycin activation in three commercial IVF media and subsequent culture in similar culture media (KSOM/COOK Blastocyst medium). No significant differences were observed in 2-cell and blastocyst formation rates among all three different IVF media used during activation (10 minutes) (Table 4). However, Vitrolife medium resulted in a significantly reduced blastocyst formation rate

compared to the KSOM ($P < 0.05$) and strontium control ($P < 0.01$) (Table 4). Furthermore, a significantly reduced blastocyst formation rate was observed in SAGE medium compared to the strontium control ($P < 0.01$, Table 4). Additionally, during parthenogenetic activation CC medium resulted in comparable 2-cell and blastocyst formation rate when compared to the KSOM and strontium control ($P < 0.01$, Table 4).

Table 4. The activation rate and subsequent embryonic developmental potential of mouse oocytes following exposure to different culture media supplemented with varying concentrations of ionomycin

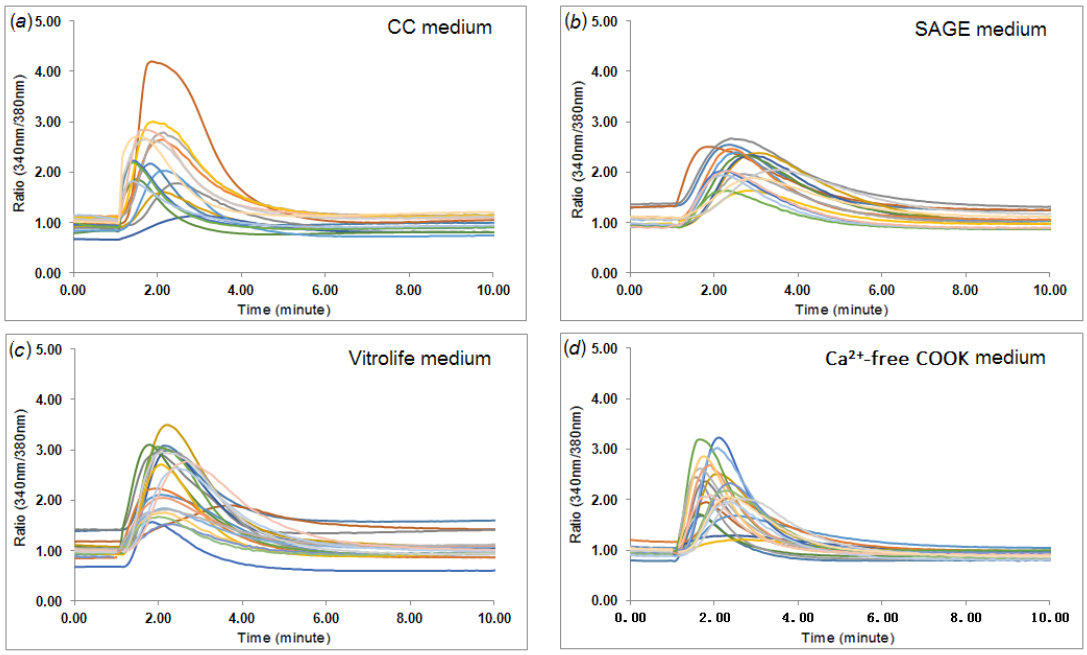
	No. oocytes	2-cell rate n (%)	Blastocyst rate n (%)
1x[Ca^{2+}] KSOM+5μM ionomycin	18	8 (44)[a]	0[c,d]
1x[Ca^{2+}] KSOM+10μM ionomycin	20	14 (70)[a]	11 (79)
1x[Ca^{2+}] KSOM+15μM ionomycin	18	12 (67)[a]	5 (42)[c]
Ca^{2+}-free KSOM+10μM ionomycin	20	5 (25)[b]	0[c]
3x[Ca^{2+}] KSOM+10μM ionomycin	18	12 (67)[a]	9 (75)
6x[Ca^{2+}] KSOM+10μM ionomycin	19	13 (68)[a]	6 (46)[c]
CC medium+10μM ionomycin	46	26 (57)[a]	17 (65)
SAGE medium+10μM ionomycin	23	16 (70)[a]	7 (44)[c]
Vitrolife medium+10μM ionomycin	28	20 (71)[a]	7 (35)[c,d]
Ca^{2+}-free KSOM+10mM Sr^{2+}	20	20 (100)	18 (90)

Data represent numbers (percentage). [a]$P < 0.05$ when compared to 2-cell rate of 10mM Sr^{2+} medium, [b]$P < 0.05$ when compared to 2-cell rate of 10μM ionomycin with 1x[Ca^{2+}] KSOM medium, [c]$P < 0.05$ when compared to blastocyst rate of 10mM Sr^{2+} medium, [d]$P < 0.05$ when compared to blastocyst rate of 10μM ionomycin with 1x[Ca^{2+}] KSOM medium.

Ionomycin dissolved in three different commercial IVF media influence the Ca^{2+} response of human oocytes

Our study included a total of 75 oocytes (25 GV-MII 24 hours, 20 MI-MII 3 hours and 30MI-MII 24 hours) from 33 patients, allocated to the different groups. The Ca^{2+}-release patterns of the oocytes were investigated following exposure to 10μM ionomycin dissolved in CC medium, SAGE medium, Vitrolife medium and Ca^{2+}-free COOK medium. The allocations of GV and MI oocytes across the commercially available culture media were analyzed by the Chi-square test, and no significant difference was observed ($P = 0.175$). Moreover, the overall mean age of the patients was 32.4±4.4 years, ranging from 22 to 43 years old. We performed an additional ANOVA test to compare the age of the patients across these four groups, and no significant difference was observed ($P = 0.327$, data not shown).

Fig. 2. Ca^{2+} response of IVM human oocytes following ionomycin exposure combined with different IVF medium.



Each trace represented for a human oocyte.

Following the exposure of human oocytes to ionomycin, the intracellular Ca^{2+} level rapidly increased as observed in mouse oocytes. The single Ca^{2+} rise was characterized by a rapid upstroke followed by a slower decline towards the baseline (Fig. 2). Overall, the RA and AUC of Ca^{2+} rise of human oocytes were significantly lower compared to mouse oocytes across all observed commercial media ($P < 0.001$). The time to peak of the triggered Ca^{2+} rise was significantly higher in the SAGE group when compared to the Ca^{2+} rise induced in the other three groups (Table 5). The RA of Ca^{2+} flux induced by ionomycin did not differ among the commercial media groups (Table 5, Fig. 2). Interestingly, we observed that the AUC of the Ca^{2+}-rise tended to increase concomitantly with an increase in the amount of total calcium present in the medium (Table 5). However, a significantly higher AUC was only observed in oocytes exposed to ionomycin dissolved in Vitrolife compared to Ca^{2+}-free COOK medium (Table 5).

Table 5. The impact of calcium concentration on the Ca^{2+} rise of IVM human oocytes, comparison of three different commercially available IVF culture media.

	No. oocytes	Time to peak (minute)	RA of the Ca^{2+} rise	AUC of the Ca^{2+} rise	Calcium concentration (mM)
CC	16	1.88±0.43[a]	1.38±0.65	2.51±1.52	1.70 ± 0.01
SAGE	16	2.54±0.40	1.11±0.25	2.87±0.92	2.75 ± 0.12[c]
Vitrolife	21	2.16±0.40[a]	1.42±0.57	3.13±1.09[b]	2.82 ± 0.03[c]
Ca^{2+}-free	22	2.03±0.34[a]	1.26±0.55	1.99±0.75	NA

Data represent the mean ± s.d. [a]$P < 0.05$ when compared to time to peak of SAGE medium, [b]$P < 0.05$ when compared to AUC of Ca^{2+}-free medium, [c]$P < 0.05$ when compared to Calcium concentration of CC medium. RA, relative amplitude. AUC, area under the curve. NA, not applicable.

Discussion

To date, the application of different AOA strategies to overcome fertilization failure after ICSI, has led to successful outcomes, with high rates of fertilization and pregnancies established by some groups (Heindryckx *et al.* 2005; Heindryckx *et al.* 2008; Kyono *et al.* 2009; Terada *et al.* 2009; Ebner *et al.* 2012; Montag *et al.* 2012; Kim *et al.* 2014; Vanden Meerschaut *et al.* 2014). However, the non-standardization of protocols across laboratories and numerous variables affecting the procedure have also resulted in inconsistent data. In some instances the application of AOA protocols have in fact led to reduced fertilization and pregnancy rates, or no improvement in outcomes when compared to routine ICSI (Borges *et al.* 2009b; Borges *et al.* 2009a; Ebner *et al.* 2012; Montag *et al.* 2012). In the present study, we investigated specific external parameters, which may potentially affect outcomes following the application of the most well-established AOA techniques.

In mouse oocytes, both the Ca^{2+}-oscillatory pattern and the embryonic developmental potential were affected by the concentration of ionomycin and the [Ca^{2+}] in the culture medium during AOA. Suboptimal conditions during the application of AOA even in a short time interval of only 10 minutes, had major effects on the subsequent oocyte activation rate and more importantly hampered embryonic developmental potential. These effects were directly associated with [Ca^{2+}] and ionomycin concentrations in the medium used for AOA. We therefore reveal, for the first time, that the Ca^{2+}-oscillatory pattern triggered by ionomycin of IVM human oocytes may be influenced by the type of IVF culture medium used during the AOA procedure. Our results suggest that this effect is likely correlated with the total calcium amount in the culture medium. These important new findings should be carefully considered when applying AOA protocols to restore fertilization rates in human. We further encourage IVF laboratories to define more standardized protocols for AOA and account for the possible effects of parameters such as the

culture media used during the AOA procedure.

Ca^{2+} signaling serves as a universal clue for the completion of meiosis and initiation of embryo development (Ramadan *et al.* 2012; Wakai *et al.* 2013). The Ca^{2+} flux during ionomycin exposure is recorded as a single Ca^{2+} transient pattern (Swann and Ozil 1994; Gualtieri *et al.* 2011), which was also observed in both our human and mouse oocytes. During *Xenopus* and starfish oocyte activation, ionomycin predominantly induces Ca^{2+} release from ER by targeting IP_3 sensitive stores (Yoshida and Plant 1992; Vasilev *et al.* 2012). By exposing oocytes to ionomycin in Ca^{2+}-free conditions, we provide further insights into the underlying mechanisms of ionomycin and its function in mammalian oocyte activation. In Ca^{2+}-free medium, the observed Ca^{2+} rise originated mainly from the endoplasmic reticulum (ER). It was initiated immediately after exposure and reached a comparable RA, at the same time point, as the Ca^{2+}-containing groups in both human and mouse oocytes. However, the AUC was significantly reduced in oocytes activated in Ca^{2+}-free conditions, suggesting that ionomycin mediates Ca^{2+} release from both the IP_3R and the surrounding culture medium in mouse and human oocytes.

In mice, we found that AOA with ionomycin in Ca^{2+}-free media led to a complete failure in blastocyst formation. Remarkably, Ca^{2+}-free KSOM was used only during the 10-minute time interval of AOA and the subsequent embryo culture was performed in Ca^{2+}-containing KSOM. Our results show that the type of culture medium used during the short process of AOA during ten minutes can affect not only the activation rate, but more importantly also impact further embryonic development in mouse. It is important to take into account that the *in vitro* culture of the mouse oocytes was performed in the SAME culture system (KSOM/Cook Blastocyst) in all the experimental groups. Therefore, the observed effect is only due to this small-time frame of artificial oocyte activation during 10 minutes in the various conditions. Importantly, the Ca^{2+}-free medium could contain some Ca^{2+} (~10 µM) due to the contamination from the recipients in which the solution is stored. Therefore, the contribution and proportion of extra- and intracellular Ca^{2+} during ionomycin activation requires precisely investigation, for instance the utility of BAPTA-MA (chelation of intracellular Ca^{2+}).

Moreover, when subjecting oocytes to ionomycin with different human culture media, human oocytes showed an overall delayed time to peak (the speed of Ca^{2+} release through ER) and reduced time of the transient dropping from the peak to baseline (the speed of removing the free cytosolic Ca^{2+}) compared to mouse oocytes (Wakai *et al.* 2013). Under the conditions of the current study, it may indicate that in human, ionomycin induces Ca^{2+} signaling mainly through intracellular stores, whereas in mouse, the influence of the surrounding environment is more substantial.

Given the limited availability of human oocytes for research, we tested the influence of ionomycin concentrations and [Ca^{2+}] in the culture media on the Ca^{2+} oscillatory pattern and embryonic development potential in mouse oocytes. Our results indicate that Ca^{2+} signaling induced by 5µM ionomycin was not sufficient to allow for the critical threshold of Ca^{2+} signaling proposed by *Toth et al.* (Toth *et al.* 2006) to be reached. This resulted in adverse downstream effects on activation rate and blastocyst formation. Furthermore, our data reveal that higher concentrations of ionomycin, such as 15µM do not benefit oocyte activation rates. Although, applied during AOA (Ebner *et al.* 2012), such high concentrations of ionophore may have a negative impact on further embryonic development as shown in our mouse model. Therefore, inconsistent fertilization rates after AOA in human, may also be attributed to the varying concentrations of ionophore used, as reported concentrations range from 5µM to 15µM (Moaz *et al.* 2006; Ebner *et al.* 2012; Montag *et al.* 2012). Moreover, several studies have reported that ionomycin is more potent and specific than calcimycin (A23187) during oocyte activation at the same concentration in various species (Kauffman *et al.* 1980; Vasilev *et al.* 2012; Nikiforaki *et al.* 2016). As such, the impact of high ionomycin concentrations on subsequent embryonic development may be more profound, in turn directly affecting the efficiency of AOA.

Supplementing the medium with a higher Ca^{2+} concentration during AOA increased the activation rates and further embryonic development of bovine and porcine oocytes (Miyoshi *et al.* 2008; Fernandes *et al.* 2013). Similarly, enhanced fertility in micro-inseminated mouse oocytes with a higher [Ca^{2+}] IVF medium was described before (Fujimoto *et al.* 1994). In contrast, we did not find any improvement in either the Ca^{2+}-oscillatory pattern nor mouse embryonic developmental potential after ionomycin exposure in the presence of higher [Ca^{2+}] (3x) in the culture media. Conversely, our results showed a lower blastocyst formation rate with a higher rate of fragmentation in 6x[Ca^{2+}] medium compared to other KSOM groups. Ca^{2+} transport during a rise in Ca^{2+} requires the action of the sarco-ER Ca^{2+}-ATPases, plasma membrane Ca^{2+}-ATPases, as well as the Na^{+}/Ca^{2+} exchanger (Berridge *et al.* 2000; Bootman *et al.* 2001). The reduced embryonic development observed, may be attributed to inhibition or damage of the Ca^{2+} pump of the ER and plasma membrane, propagated by the high concentrations of intracellular Ca^{2+} through both specific transport and the non-specific channel after ionomycin assisted Ca^{2+} permeability (Mason and Grinstein 1993). This phenomenon could result in the dysfunction of intracellular Ca^{2+} regulation and hence influence the subsequent embryonic developmental potential.

When comparing the AOA efficiency using ionomycin in combination with different commercial IVF media, we found the AUC of Ca^{2+} rise in human oocytes increased slightly with the concentration of the total calcium in the medium. However, in mouse oocytes, the Vitrolife medium containing a higher total calcium concentration, generated a significantly lower AUC during ionomycin exposure. Importantly, the blastocyst formation rate was significantly

reduced when AOA was performed in Vitrolife medium, despite using similar culture media to support mouse embryonic development. Conversely, CC medium resulted in similar activation and blastocyst rates compared to controls. As the Ca^{2+} pattern of mouse oocytes might be influenced by some other culture medium components, the embryonic development potential test in mice represents a better potential methodology to study the influence of commercial culture medium on human oocytes.

Apart from being the result of varying concentrations of calcium, the altered Ca^{2+} pattern and blastocyst rate in mouse embryos could also be attributed to the variability of proteins, other ions or EDTA present in the commercial human embryo culture media (Morbeck *et al.* 2014a; Morbeck *et al.* 2017). Although the variation in composition of protein supplements in the medium could influence the blastocyst developmental potential in mouse (Morbeck *et al.* 2014b), it cannot account for the observed difference, as similar concentrations of HSA (5mg/mL) are supplied to all human media. The concentration of external magnesium ions (Mg^{2+}) may however play a role, as increased concentrations (2.5mM) of external Mg^{2+} has shown to inhibit the intracellular Ca^{2+} release pattern of sea urchin egg (Graeff et al. 1995), whereas oocyte fertilization in medium with reduced concentrations of Mg^{2+} (0.2mM) significantly improves blastocysts rates in both mouse and human (Herrick et al., 2015). To our knowledge, the concentrations of Mg^{2+} in the observed media varied from 1.5mM (CC medium) to unknown (assumed around 1.7-1.8mM, similar to other media from the same distributor), analyzed by colorimetric methodology (Morbeck *et al.* 2014a; Morbeck *et al.* 2017). Moreover, the altered Ca^{2+} pattern could also result from the undisclosed concentrations of the membrane-impermeable metal ion chelator EDTA, as EDTA impairs embryonic developmental potential at high concentrations (>5mM) (Berridge et al., 2000; Lee et al., 2007). Further investigations are required to verify the effect of these components.

Due to the lack of available human oocytes for research, it was not possible to evaluate the effect of different amounts of Ca^{2+} and other components present in the culture medium during artificial activation on embryonic developmental potential. However, reports performing AOA in CC conditions have resulted in more optimal outcomes (Heindryckx *et al.* 2008; Vanden Meerschaut *et al.* 2014). Our results show that the type of culture medium used during the process of AOA can affect not only the activation rate, but more importantly also impact further embryonic development in mouse. Therefore, we recommend that clinics applying AOA technology should take this into account. Currently, more standardized protocols that synchronize the crucial components of AOA medium are warranted.

In conclusion, we have demonstrated that both the concentration of ionomycin and [Ca^{2+}] in the culture medium directly affects the procedure of AOA in mouse oocytes. Importantly, mouse embryonic developmental potential was significantly influenced by the concentration of

ionomycin and [Ca^{2+}] in the surrounding culture medium. Exposure to suboptimal culture conditions during the crucial activation phase, even during this short time interval, not only has detrimental effects on oocyte activation, but also hampers downstream processes such as embryonic developmental potential. In addition, our data also indicated that ionomycin acts on both Ca^{2+} stores of ER and Ca^{2+} from the surrounding medium in oocytes. As such, these parameters should be considered when optimizing or applying AOA protocols. Further studies are required to determine the optimal ionomycin and Ca^{2+} concentrations for human oocytes and verify their effect on the Ca^{2+}-oscillatory potential, activation efficiency and embryonic developmental competence.

Authors' Note

Y.L., B.H., L.L. and P.D.S. conceived and designed the study. Y.L., D.B., and M.F.B performed experiments reported in Tables 1-4 and Figures 1-3. Y.L., B.H., M.P. wrote the manuscript and assembled the figures. M.V.d.J. assisted in the English editing of the manuscript. All authors contributed to the interpretation of the results and the English editing of the manuscript.

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Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Chapter 5

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

Objective

Although strontium (Sr^{2+}) is used as an agent in assisted oocyte activation (AOA), to overcome fertilization failure after ICSI in the clinic, its efficiency is still deemed controversial. The transient receptor potential ion channels, vanilloid 3 (TRPV3), have recently been reported to mediate Sr^{2+} induced mouse oocyte activation. Therefore, in the third study of this thesis, we investigated the activation efficiency of Sr^{2+}.

Conclusion

Sr^{2+} did not promote Ca^{2+} oscillations or provoke activation in human oocytes. However, transcripts of TRPV3 channels were present in *in vitro* matured (IVM) MII human oocytes. The pattern of localization of TRPV3 protein in human oocytes differed from what has been reported in mouse oocytes. Both agonists of TRPV3, 2-aminoethoxydiphenyl borate (2-APB) and carvacrol, promoted a single Ca^{2+} rise and activated more than half of the exposed human oocytes. Thus, the benefit of clinical application using Sr^{2+} to overcome fertilization failure after ICSI requires further validation.

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

Running title: Strontium cannot active human oocytes

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Study question: Are the transient receptor potential ion channel vanilloid 3 (TRPV3) channels present and functional to mediate strontium (Sr^{2+}) induced artificial activation in human oocytes as was recently reported in mouse?

Summary answer: Sr^{2+} is not able to induce Ca^{2+} rises and provoke activation in human oocytes, however, TRPV3 channels were present in *in vitro* matured (IVM) MII human oocytes at transcription level and are shown to be functional upon using TRPV3 agonists inducing Ca^{2+} rises and oocyte activation.

What is known already: Selective activation of TRPV3 by agonists provokes 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 clinic, but its efficiency is still controversial and the mechanism how it mediates the Ca^{2+} flux has not been studied yet in human.

Study design, size, duration: The protein distribution and the mRNA expression of the TRPV3 channels was investigated in *in vitro* matured (IVM) metaphase II (MII) and in *in vivo* matured (IVO) MII human oocytes. The Sr^{2+} and TRPV3 agonists induced Ca^{2+} response and subsequent embryonic developmental potential was further analysed in both mouse and human oocytes.

Participants/materials, setting, methods: MII oocytes from B6D2F1 mice as well as IVM human oocytes and IVO oocytes with aggregates of smooth endoplasmic reticulum clusters (SER) were used. The expression of TRPV3 protein was determined by confocal microscopy and Reverse Transcription PCR, the temporal evolution of intracellular Ca^{2+} concentration was measured by time-lapse imaging after exposure to Sr^{2+} and TRPV3 agonists. Artificial activation efficiency was assessed using these agents.

Main results and the role of chance: Sr^{2+} could not promote Ca^{2+} oscillations or provoke activation in human oocytes. Transcripts of TRPV3 channels were present in *in vitro* matured (IVM) MII human oocytes. The pattern of localization of TRPV3 protein in human oocytes differed from the one reported in mouse oocytes. Both agonists of TRPV3, 2-aminoethoxydiphenyl borate (2-APB) and carvacrol promoted a single Ca^{2+} transient and activated more than half of exposed human oocytes.

Large scale data: 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 interest declared.

Keywords: assisted oocyte activation, Sr^{2+}, TRPV3 channels, Ca^{2+} rise, human oocytes

Introduction

Mammalian oocyte activation is triggered by the sperm factor phospholipase C zeta (PLCζ), which initiates a series of oscillations of Ca^{2+} levels within the ooplasm (Saunders *et al.*, 2002; Tesarik, 2002). PLCζ 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, the 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 the characteristic Ca^{2+} oscillation pattern in 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 in the oocyte activation mechanism are associated with fertilization failure or low fertilization rate following the treatment of intracytoplasmic sperm injection (ICSI) (Rawe *et al.*, 2000; Yanagida, 2004; Swain and Pool, 2008; Neri *et al.*, 2014). The activation potential of sperm from patients who experienced fertilization failure can be evaluated by 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 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 studies, in which repetitive Ca^{2+} transients are observed (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 generally modulated by various stimuli and ligands, including natural compounds like 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+} convinced to be the most effective AOA method in a mouse model with deficient sperm activation capacity (Vanden Meerschaut *et al.*, 2013) or more recently in a knockout mouse model for PLCζ (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+} rise and oocytes activation, as well as the expression and functionality of the TRPV3 channels in human oocytes. In addition, the use of TRPV3 channels 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 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 administrated with a GnRH agonist (Decapeptyl; Ferring) or antagonist (Cetrotide;Merck Serono). Ovarian stimulation was performed by administering hMG (Menopur; Ferring) or recombinant FSH (Gonal-F; Merck Serono) at a dose of 112.5-300IU daily and ovulation was induced with 5000IU hCG (Pregnyl;MSD). Oocytes were enzymatically denuded by brief exposure to 80IU/ml hyaluronidase (Irvine Scientific), 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 MII stage (presence of a polar body and absence of a GV structure). Donated GV oocytes were further cultured in medium 199, supplemented with 10ng/ml epidermal growth factor, 1mg/ml estradiol, 10mIU/ml recFSH, 0.5mIU/ml hCG, 1mM l-glutamine, 0.3mM sodium pyruvate, 0.8% (v/v) human serum albumin (Red Cross, Belgium), 100IU/ml penicillin G and

100 mg/ml streptomycin sulfate at 37°C in 6% CO_2 and 5% O_2 for 24h. Immature MI stage oocytes were cultured in Sydney IVF COOK cleavage (CC) medium (Cook Ireland Ltd) for 3h or 24h based on the 1st polar body extrusion. 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 concern at our Department, due to the earlier reported malformations and impaired pregnancy outcomes (Otsuki *et al.*, 2004; Sa *et al.*, 2011; Itoi *et al.*, 2015). 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 I, $P>0.05$).

Source and culture of mouse oocytes

6-10-week-old B6D2F1 hybrid female mice were stimulated with 7.5IU pregnant mare serum gonadotrophin (PMSG, Folligon®, Intervet, Boxmeer, The Netherlands), followed by 7.5IU human chorionic gonadotrophin (hCG, Chorulon®, Intervet, Boxmeer, The Netherlands), 46-48h later. MII oocytes were harvested 12-14h following hCG injection in HEPES buffered potassium simplex optimized medium (KSOM-HEPES) supplemented with 4mg/ml bovine serum albumin (BSA, Calbiochem). 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 the exposure to Sr^{2+} and TRPV3 agonists

Human and mouse oocytes were loaded with 7.5μM of the radiometric Ca^{2+} sensitive dye Fura-2 acetoxymethyl (AM) ester (Invitrogen, Life Technologies Europe B.V., Belgium) at 37˚C in 6% CO_2, 5% O_2 and for 30min and then washed extensively. Subsequently, oocytes were placed in 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 20x objective. Fluorescence was recorded at an emission wavelength of ~510nm every 5s. The ratio of both Ca^{2+} induced signals (340nm/380nm) 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 and SERs oocytes were transferred to a drop of Ca^{2+}/Mg^{2+} free Earle's Balanced Salt Solution (EBSS) supplemented with 10mM $SrCl_2$ (Life Technologies). The Ca^{2+} images were recorded every 5s for a duration of 6h. Following the first 2h of exposure, a group of human oocytes (n=5) that did not respond to the Sr^{2+} was subsequently exposed to 10μM

ionomycin (cat. no. I9657) dissolved in COOK Cleavage medium for 15min. 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 5s for a duration of 2h, immediately after transferring the eggs to a drop of Ca^{2+}-free KSOM with 10mM $SrCl_2$, 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 that exposed to 200μM 2-APB or 200μM carvacrol within 1% PVA supplemented albumin-free IVF™ medium (Vitrolife, Göteborg, Sweden) for 30min. While mouse oocytes were subjected to the same concentration of the agonists but supplemented in 1% PVA KSOM (BSA-free) medium for 30min. The stock solutions of TRPV3 agonists were prepared by dissolving them in Dimethyl Sulphoxide (DMSO), as done for ionomycin in our clinic (Heindryckx *et al.*, 2008). The amount of DMSO added to the activation medium was tested and proven not capable of inducing Ca^{2+} rise or activation of mouse oocytes.

All oocytes were distributed randomly across the groups and tested within 2 hours after assessing the maturation state. A maximum of 3 oocytes were measured simultaneously. Baseline drifting was adjusted before retrieving values for amplitude (value at maximum increase in fluorescence intensity per peak) expressed in arbitrary units (AU). Frequency reflected the total number of oscillations per recording period. Area under the curve (AUC) of the Ca^{2+} rise was calculated and expressed in AU x minutes.

Parthenogentetic activation of MII human and mouse oocytes

To test the activation efficiency of 10mM 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+} in the duration of 4h. For creating diploid parthenogenetic embryos, 2μg/ml Cytochalasin D (CCD) was supplemented in both of the activation media. In the agonists activation tests, human oocytes were treated with 200μM 2-APB for 30min or 200μM carvacrol for 10min dissolved in IVF medium at 37˚C, while mouse oocytes were exposed at the same conditions as aforementioned, but within KSOM medium (albumin-free 0.1% PVA) (Carvacho *et al.*, 2013). CCD was supplemented to the subsequent culture medium for 4h. Following activation, human oocytes were further cultured in CC medium for 16h, and mouse oocytes were incubated in KSOM for 60-72h followed by an 24h extended culture in COOK Blastocyst medium. The activation of human oocytes was evaluated by a single pronuclear formation (1PN) 16 hours post treatment. Mouse embryo development was assessed at 24 hours (two-cell), 72 hours (morula/early blastocyst) and 96 hours (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, 1% BSA-supplemented PBS and fixed in PBS-BSA containing 2% paraformaldehyde for 45 minutes at room temperature. Oocytes were washed and blocked in PBS containing 0.1M glycine, 2% goat serum and 0.01% Tween 20 for at least one hour. Oocytes were then permeabilized with 0.1% Triton X-100 for 15 minutes at room temperature. Subsequently, samples were washed with PBS supplemented with 2% goat serum and 1% poly-vinyl alcohol (PVA) followed by a incubation at 4ºC with the primary antibody against TRPV3 (1:100, 10 µg/ml, Neuromab, 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 one hour at room temperature, followed by extended washings. In addition, chromosomes were stained with Ethidium homodimer-2 (1:500, Life Technologies, USA) for an hour at room temperature. The negative controls were treated with the secondary antibodies alone. Finally, the oocytes were mounted in Mowiol containing 0.01% phenylenediamine and imaged using a laser scanning microscope, Nikon A1R confocal microscope (Nikon Instruments, Paris, France) with a 60× Plan Apo VC oil immersion objective. The TRPV3 distribution and chromosome alignments were obtained from Z-stacks (0.5-0.75µm/Z-step), using ImageJ software.

Reverse Transcription PCR on pooled IVM human oocytes

RNA was extracted from two groups of pooled IVM MII and IVO SERs human oocytes (n=19 in total, Table I), using PicoPure RNA isolation kit as described by the manufacturer (Life Technologies, USA). The following cDNA synthesis was performed using the Superscript VILO cDNA synthesis kit (Invitrogen, USA). Primers were designed to amplify the fragment spanning Exon6 and Exon7 of TRPV3 (Forward AGGCTTCTACTTCGGTGAGAC, Reverse AGGGCGTGAAGGATGTTGTTG) using Primer3 program.

The Reverse Transcription Polymerase Chain Reaction (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 5min followed by 35 cycles of denaturation at 94°C for 30s, annealing at 64°C for 30s, extension at 72°C for 45s and a final extension at 72°C for 10min. The RT-PCR products were subsequently loaded on to the fragment analyzer and analyzed using PROsize program. Subsequently, the RT-PCR products was purified using Exo-AP and bi-directional Sanger sequencing was performed on the purified RT-PCR products using Big Dye Terminator kit (ABI, USA). The Sanger sequencing products were further cleaned using magnetic beads with 85% alcohol and analyzed by 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.

Results

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

To verify the Ca^{2+} oscillatory and activation ability of Sr^{2+} exposure, IVM and IVO MII human oocytes (n=25, Table I) were subjected to 10mM 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 6h (Figure 1A showed records from the first 2h). In contrast, mouse oocytes exposed to the same concentration of Sr^{2+} showed dynamic intracellular Ca^{2+} oscillations during the 2h analysis (Figure 1B). To test the reactivity to Ca^{2+} triggers of the human oocytes, 5 IVM human oocytes were further subjected to 10μM ionomycin following a 2h exposure of Sr^{2+}. All 5 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 (Figure 1C, Table II). Activation capacities of the human oocytes (n=10) were further evaluated, following Sr^{2+} exposure. None of the human oocytes showed 1 PN after 16h following Sr^{2+} exposure, in contrast, all of the mouse oocytes activated and cleaved, 16h post Sr^{2+} activation (Table III).

Table I The allocations of IVM and IVO (SERs) human oocytes across all studying groups.

		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%)	a/n
RT-PCR	Group I	11	3 (27%)	8 (73%)	a/n
	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%)

a/n, not applicable.

Table II The Ca^{2+} response of human oocytes following the exposure to varied 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	a/n	a/n
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}

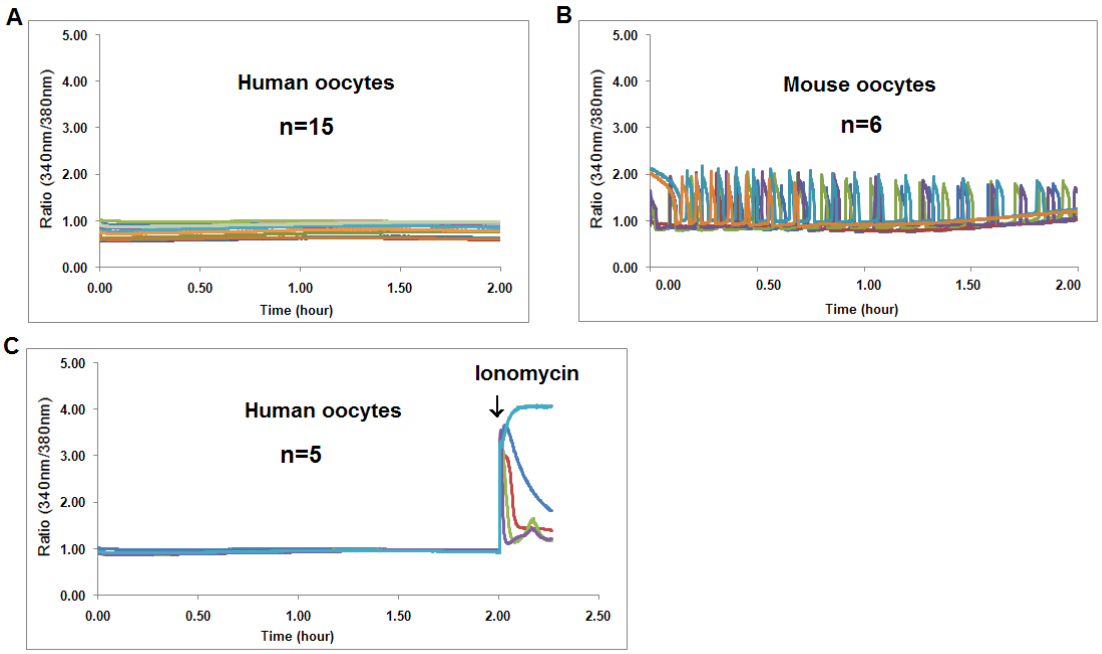
ANOVA and Bonferroni's Multiple Comparison Test $^{a}P<0.01$, $^{b,c,d}P<0.05$, a/n, not applicable. Values with same superscripts differ significantly.

Table III The response of human and mouse oocytes following exposure to Sr^{2+}, 2-APB and carvacrol

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

Chi-square and Fisher's exact test: $^{a,b,d,e}P<0.01$, $^{c}P<0.05$, a/n, not applicable. Values with same superscripts differ significantly.

Figure 1. Sr^{2+} is not capable of provoking Ca^{2+} rise in human oocytes.

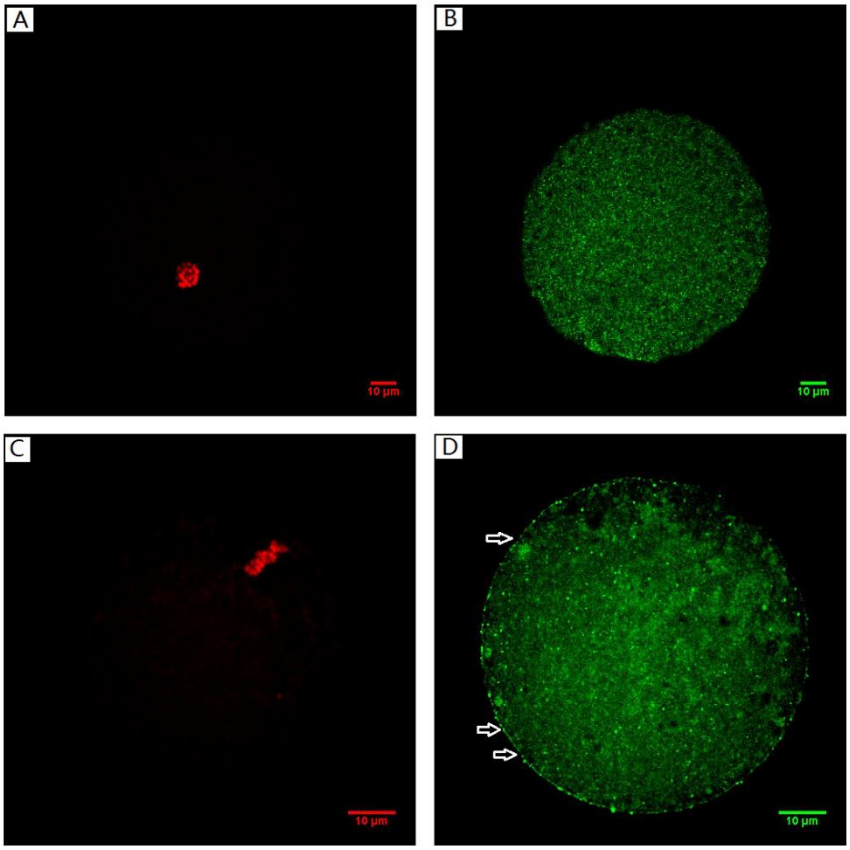


(A) Sr^{2+} cannot induce Ca^{2+} rise of human MII oocytes during 2 hours of measurement. (B) Mouse MII oocytes exhibit repetitive Ca^{2+} transients following the exposure to 10mM Sr^{2+}. (C) Exposure of human oocytes to ionomycin following Sr^{2+} incubation verify the reactivity of human oocytes to another AOA trigger.

TRPV3 channels are expressed in human oocytes

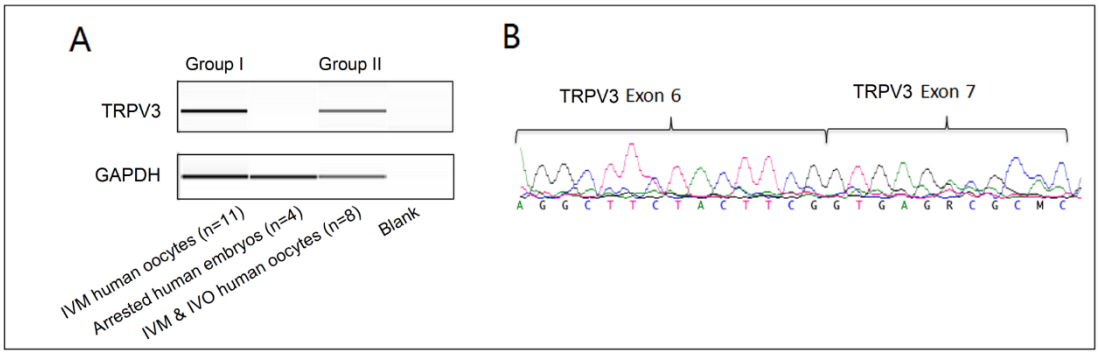
The TRPV3 channels were recently identified as the major channels to conduct Sr^{2+} influx and induce activation of mouse oocytes (Carvacho *et al.*, 2013; Lee *et al.*, 2016). Since Sr^{2+} failed to activate human oocytes or elicit Ca^{2+} transients, we further verified the expression and the function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

Figure 2. The localization of TRPV3 protein in human MII oocytes.



(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 IVO oocytes are aligned at the equatorial plate. (D) Mouse TRPV3 proteins are expressed and concentrated at the cytoplasmic membrane.

Figure 3. The TRPV3 mRNA expression in IVM MII and IVO SER human oocytes.

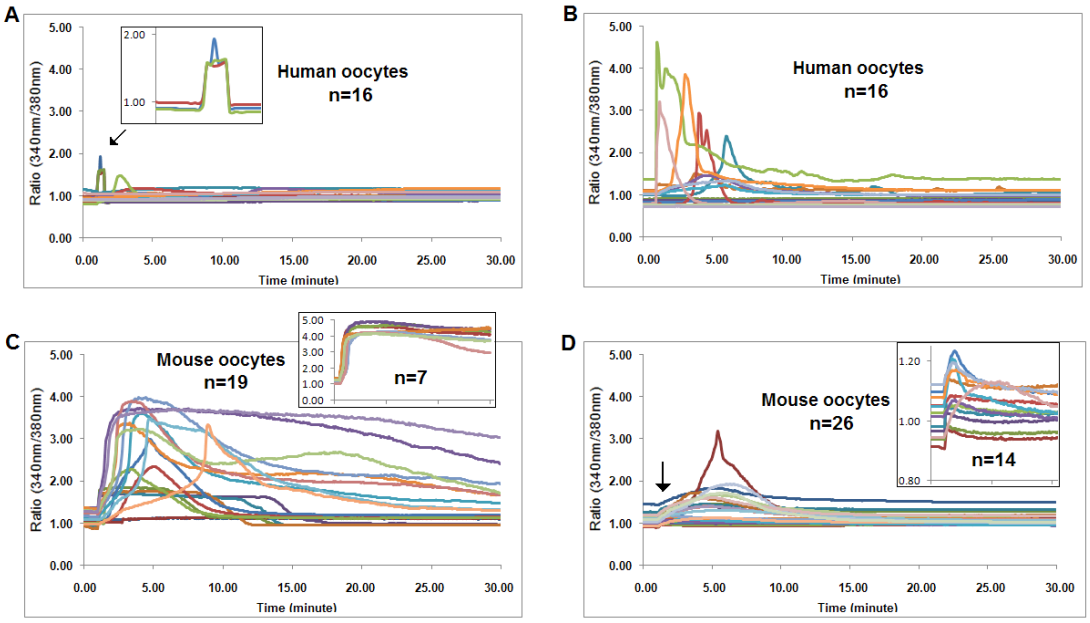


(A) The transcripts of TRPV3 are expressed in two groups of pooled IVM and IVO human oocytes. (B) Chromatogram showing the Exon 6-7 junction of the TRPV3 gene after sanger sequencing of the RT-PCR product.

TRPV3 agonists triggered Ca^{2+} rise and provoked oocyte activation of human and mouse oocytes

The Ca^{2+} oscillatory and activation 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 200μM 2-APB, the intracytoplasmic Ca^{2+} increased in 25% (4 out of 16) of them, with displaying a small rise during the first 5min of measurement (Figure 4A, 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 Ca^{2+} level compared to the Ca^{2+} rise triggered by 2-APB (*P*=0.0034), during the first 10min of exposure (Figure 4B, Table II). The 4 IVM oocytes which responded with much lower Ca^{2+} fluctuation (RA 0.28±0.14, AUC 1.54±0.56) were not included (Figure 4B, 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 Ca^{2+} triggers. As a result, all oocytes exhibited a sharp Ca^{2+} rise following the ionomycin exposure, showing a positive reactivity to the Ca^{2+} ionophore (Table II).

Figure 4. The TRPV3 agonists, 2-APB and carvacrol, induced Ca^{2+} response of both human and mouse MII oocytes.



(A) Increased intracellular Ca^{2+} of human MI oocytes provoked by 200μM 2-APB, a small peak exhibited in 4 human oocytes. (B) 5 out of 16 human MII oocytes respond to 200μM carvacrol, showing a sharp Ca^{2+} rise. (C) Elevated intracellular Ca^{2+} of mouse oocytes provoked by 200μM 2-APB, showing a great Ca^{2+} peak. (D) Lower Ca^{2+} response in mouse oocytes respond to 200μM carvacrol.

The addition of 200μM 2-APB to MII mouse oocytes (n=26) dramatically increased Ca^{2+} levels and immediately provoked a protracted peak during the 30min exposure (Figure 4C). Almost all mouse oocytes (n=24, 92%) responded to 2-APB, the remaining oocytes failed to show any Ca^{2+} rise (Figure 4C). The same concentration of carvacrol also evoked increases of intracellular Ca^{2+} in 46% (n=12) of the mouse oocytes showing a small peak in 10min duration of exposure (Figure 4D), whereas, 52% of which exhibited a small Ca^{2+} rise fluctuation (absolute amplitude less than 0.2) in the first 5min of exposure (Figure 4D).

Furthermore, the activation potential of human and mouse MII oocytes was investigated as well, following the application of these 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 activation tests. Interestingly, more than half of the human oocytes activated and formed 1PN, observed at 16h post stimulation (63% and 71% for 2-APB and carvacrol respectively, Table III). When exposing mouse oocytes to 200μM 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 2-cell stage compared to the 2-APB group (P<0.0001, Table III).

Discussion

To date, Sr^{2+} has been applied as an AOA method to overcome fertilization failure or low fertilization rates (Kyono *et al.*, 2008; Kim *et al.*, 2012, 2014), in 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+} rise 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 PLCζ at fertilization. As such, Sr^{2+} has been applied initially as an AOA agent in clinic to overcome fertilization failure after ICSI. Consequently, improved fertilization rates and embryo qualities were reported from several couples with repetitive fertilization failure (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 Ca^{2+} increase or provoking activation of human oocytes. These discrepancies could be due to the lack of diagnostic methods used in those studies, to show a real sperm-related activation deficiency in those reports (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 globozospermic patients can achieve successful fertilization even after the application of routine ICSI in the absence of AOA (Huang *et al.*, 2010). Therefore, it is hard to illustrate whether the patients enrolled in those AOA studies using Sr^{2+} as the activating agent, really required AOA in first instance, as diagnostic evidences (heterologious ICSI, genetic screening of PLCz, Ca^{2+} pattern analysis) were not obtained to show a lack of activation capacity in their sperm.

To enable activation of mouse oocytes, extracellular Sr^{2+} is permeating through its functional membrane channel, TRPV3 (Carvacho *et al.*, 2013), promoting downstream oscillations in $[Ca^{2+}]i/[Sr^{2+}]$ of the oocytes, probably by sensitizing IP3Rs and thus facilitating Ca^{2+} oscillations (Zhang *et al.*, 2005), or substituting for Ca^{2+} in the potentiation of IP3Rs (Girard and Clapham, 1993; Marshall and Taylor, 1994; Lee, 2016). Thus, the failure of activating human oocytes by Sr^{2+} alone, could be attributed to the absence of membrane TRPV3 channels, which was proven not the case in the present study, or unlikely the inability of residues to conduct Sr^{2+} (Latorre *et al.*, 2007), as well as the insensitivity of IP_3R1 in responding to Sr^{2+} in human oocytes. Further investigations are required to verify it.

Furthermore, we did confirm that the transcripts of TRPV3 channels were expressed in IVM human oocytes. The TRPV3 channels were recently demonstrated to be expressed in pooled fresh IVO human oocytes by a whole transcriptome analysis as well, albeit registered low levels (Kocabas *et al.*, 2006). The diffused cytoplasmic localizations of TRPV3 protein might contribute to the observed inability of conducting Sr^{2+} influx by TRPV3 or the aberrant Ca^{2+} signaling following 2-APB treatment, owing to trafficking failure of 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 method that was used in the present setting. More future analysis, for instance, 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 Sr^{2+} influx or the conducted Sr^{2+} was not sufficient to promote Ca^{2+} release from the ER, they were shown to be functional in supporting agonists triggered Ca^{2+} rise and oocyte activation in both mouse and human oocytes. In the view of the efficiency of activating rodent oocytes, 2-APB was suggested as a potential AOA agent targeting at TRPV3 channels (Lee, 2016; Lee *et al.*, 2016). However, in our view, it is immature to encourage it at this moment, as both 2-APB and

carvacrol activates multiple Ca^{2+} 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 Ca^{2+} influx exclusively from the external culture medium (Xu *et al.*, 2006; Lee *et al.*, 2016), whereas, Ca^{2+} ionophore promoted Ca^{2+} increase from both the ER and extracellular Ca^{2+} influx (processing data from our group). Currently, ionomycin is still the most recommended agent to overcome fertilization failure after ICSI (Heindryckx *et al.*, 2005; Vanden Meerschaut *et al.*, 2014; Nikiforaki *et al.*, 2016), due to its high efficiency in provoking Ca^{2+} rise and inducing activation as demonstrated in both mouse and human oocytes (Vanden Meerschaut *et al.*, 2014; Nikiforaki *et al.*, 2016).

Author's 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 Table I-III and Figure 1-2,4. R.R. performed the RT-PCR and Sanger sequencing leading to Figure 3. Y.L., B.H., M.F.B wrote the manuscript 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 manuscript.

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Conflict of interest

None declared.

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Chapter 6

6. Distinctions in Ca^{2+} oscillatory patterns of post-ovulatory aged and reproductive-aged mouse oocytes following parthenogenetic activation

Objective

In the present study, we are investigating the differences in Ca^{2+} oscilation patterns in zygotes resulting from post-ovulatory aged and reproductively aged oocytes, as well as their embryo developmental potential following Sr^{2+} exposure.

Conclusion

We found variable disruptions in the Ca^{2+} oscillation pattern during activation of the post-ovulatory aged IVO oocytes and reproductive-aged IVM and IVO oocytes. The altered Ca^{2+} oscillation pattern of post-ovulatory aged IVO oocytes, further impaired embryo development. However, this was not the case in reproductively aged IVM and IVO mouse oocytes. Furthermore, by extending the *in vitro* culture time to 20-24 hours post GVBD, Ca^{2+} oscillatory ability and further blastocyst development were restored in IVM mouse oocytes with impaired Ca^{2+} oscillatory ability.

Title

Distinctions in Ca^{2+} oscillatory patterns of post-ovulatory aged and reproductive-aged mouse oocytes following parthenogenetic activation

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Abstract

Oocyte ageing, due to advanced female age (reproductive oocyte ageing) and prolonged *in vitro* culture (post-ovulatory ageing) are both factors associated with decreased fertilization capacity and poor embryo quality. Intracellular Ca^{2+}-signal is essential for successful mammalian fertilization and subsequent embryonic development. We primarily investigated the Ca^{2+}-release pattern and subsequent embryonic development potential of post-ovulatory aged and reproductive-aged mouse oocytes following artificial activation, compared to fresh collected control oocytes. In addition, we divided ageing oocytes into *in vitro* maturated (IVM) and *in vivo* matured (IVO) groups according to the oocyte maturation stage at retrieval, to further investigate the effect of IVM on the Ca^{2+}-release pattern. Overall, IVM oocytes from all groups showed significantly lower frequencies of Ca^{2+}-oscillations when compared to IVO oocytes (P<0.01). Following 4-6 hours *in vitro* ageing, IVM oocytes showed significantly higher frequency of Ca^{2+}-oscillations and blastocyst formation rates when compared to fresh IVM oocytes (P<0.01). In contrast, the frequency of the Ca^{2+}-rises of the IVO oocytes increased significantly with the time of ageing (P<0.01), coinciding with a declined relative amplitude (RA) and reduced embryonic development potential compared to the fresh IVO controls (P<0.01). When IVM oocytes were collected from 50-52 weeks reproductive-aged mice, a longer latent period and a higher frequency of the Ca^{2+}-oscillations was observed, when compared to IVM oocytes from young mice, with comparable RA. The IVO oocytes from aged mice showed no significant difference in the frequency of Ca^{2+}-oscillations compared to oocytes retrieved from young mice, but showed a decreased RA (P<0.01). In conclusion, distinct deregulations of the Ca^{2+}-signaling pattern occur in post-ovulatory aged and reproductive-aged oocytes during activation, which could be an underlying reason of impaired embryonic development. Only the IVM oocytes that mature to MII oocytes in a narrow time-frame, show adequate embryo development potential.

Keywords: Post-ovulatory ageing; Reproductive ageing; Ca^{2+}-release; embryonic development potential

Introduction

In mammals, an optimal window exists to fertilize Metaphase II (MII) stage oocytes, which is generally considered within 10h post ovulation (Marston and Chang 1964; Tarin, Perez-Albala et al. 1999). At fertilization, the sperm factor phospholipase C zeta (PLCζ) triggers specific Ca^{2+} oscillations within the ooplasm. Further downstream, binding of inositol 1,4,5-trisphosphate to its receptors (IP_3Rs) generates Ca^{2+}-release from the endoplasmic reticulum (ER) of the oocyte. The Ca^{2+}-fluxes are required for the initiation of multiple protein kinase signaling cascades (Parrington, Davis et al. 2007; Swann and Lai 2013; McGinnis, Pelech et al. 2014), further supporting successful fertilization and subsequent embryonic development.

When there is no fertilization in this adequate time frame, oocytes experience deterioration in quality referred to as 'post-ovulatory ageing' (Lord and Aitken 2013). This can occur both *in vivo* and *in vitro*, either in the oviduct of the female reproductive tract or in the *in vitro* culture medium of assisted reproduction settings. Post-ovulatory aged oocytes can still be injected to rescue for example failed fertilization in the clinic (Nagy, Joris et al. 1993), or serve as a valuable source for establishing embryonic stem cell lines after nuclear transfer (Wakayama, Suetsugu et al. 2007; Thuan, Kishigami et al. 2010). However, they exhibit numerous aberrations in their cell biology, for instance, cell cycle factors declines (Kikuchi, Naito et al. 2002), mitochondrial dysfunction (Tatone, Heizenrieder et al. 2011; Lord and Aitken 2013) and spindle-chromosomal abnormalities (Wakayama, Thuan et al. 2004).

At fertilization, deregulation of the Ca^{2+}-signaling has been observed in post-ovulatory aged oocytes, showing a higher frequency and a lower amplitude in the Ca^{2+} oscillatory pattern (Kolker, Fukuyama et al. 2003; Takahashi, Takahashi et al. 2003; Takahashi, Igarashi et al. 2009). As such, these post-ovulatory aged oocytes activate pathways of apoptosis, fragmentation and cell death, instead of supporting fertilization and normal embryonic development (Gordo, Kurokawa et al. 2002; Takahashi, Igarashi et al. 2009). The clinical applications of these aged oocytes are further associated with decreased fertilization capacity (Marston and Chang 1964; Ben-Rafael, Kopf et al. 1986; Badenas, Santalo et al. 1989) and embryo quality (Yanagida, Yazawa et al. 1998; Lord, Nixon et al. 2013), as well as increased likelihood of early pregnancy loss (Wilcox, Weinberg et al. 1998) and abnormalities in offspring (Tarin, Perez-Albala et al. 1999).

Moreover, *in vitro* maturation becomes an emerging procedure incorporated into the world of assisted reproductive technologies. Fertilizing *in vitro* matured (IVM) oocytes represents a cheaper and safer approach compared to routine *in vitro* fertilization, particularly for women suffering from polycystic ovary syndrome. However, the ability of both mouse and human IVM oocytes to release Ca^{2+} at fertilization is impaired (Cheung, Swann et al. 2000; Mann, Lowther

et al. 2010). The impact of post-ovulatory and reproductive ageing on IVM oocytes has not yet been elucidated.

Post-ovulatory ageing is a process distinct from ovarian ageing, which occurs within the ovary of females towards the end of the reproductive life, as they approach the climacteric, known as “reproductive ageing” (Bentov and Casper 2013). Ovarian ageing is associated with a decrease in the number and quality of ovulated oocytes, thus contributes to an increased incidence of infertility. Although the physiological consequences of reproductive ageing are known as increased percentage of abnormal or degenerating oocytes (Tarin, Perez-Albala et al. 2001), chromosomal aneuploidy (te Velde and Pearson 2002), and the injury of mitochondrial DNA (Keefe, Niven-Fairchild et al. 1995; Thouas, Trounson et al. 2005; Tatone 2008), their molecular changes, such as the Ca^{2+}-oscillatory patterns are not investigated.

Therefore, in this study, we assessed the strontium (Sr^{2+})-induced Ca^{2+}-oscillations in IVO and IVM post-ovulatory aged and reproductive-aged B6D2F1 MII oocytes after parthenogenetic activation. In addition, the activation rates and subsequent embryonic developmental potential was further investigated in these oocytes.

MATERIALS AND METHODS

All chemicals and reagents were purchased from Sigma Chemical Co. (Bornem, Belgium), unless otherwise indicated.

Ethical Approval

The mice from B6D2F1 hybrids (Iffa Credo, Brussels, Belgium) were housed and bred according to the national legislations for animal care. All procedures involving animal handling were approved by the Ghent University Hospital Ethical Committee for Laboratory Animals (ECD no. 14/45).

Culture of mouse oocytes

Mouse oocytes at the germinal vesicle (GV) stage were isolated from the ovaries of young females (8- to 10-weeks old) or reproductive-aged (50- to 52-weeks old) female mice, 46-48h post intraperitoneal injection of 7.5IU pregnant mare's serum gonadotropin (PMSG, Folligon®, Intervet, Boxmeer, The Netherlands). The antral follicles were punctured to release cumulus-enclosed GV oocytes into the potassium simplex optimized medium (KSOM)-HEPES medium, which contained 4mg/ml bovine serum albumin (BSA, Calbiochem, Bierges, Belgium) (Fulton and Whittingham 1978). Subsequently, the granulosa cells were removed by mechanical denudation prior to *in vitro* maturation. Oocytes with intact GV structures were selected and intensively washed in KSOM-HEPES, then transferred into Minimum Essential Medium Alpha (α-MEM) with glutaMAX™ (Invitrogen, Life Technologies, Merelbeke, Belgium),

and supplemented with 5% heat-inactivated fetal bovine serum (FBS; Gibco BRL, Life Technologies, Merelbeke, Belgium), 5µg/ml insulin, 5µg/ml transferrin, 5ng/ml selenium (BD Bioscience, San Jose, California, USA), 50 mIU/ml FSH and 50mIU/ml hCG (Puregon, Organon, Oss, The Netherlands) (Vanhoutte, Nogueira et al. 2009). Nuclear status was assessed after 2h of *in vitro* culture, after which oocytes showing germinal vesicle breakdown (GVBD) were selected and further cultured for another 16h. For *in vitro* ageing, the IVM oocytes were transferred in the droplets of KSOM containing 0.4% BSA, cultured for an additional 0, 4, 8 or 20h (i.e. 16, 20, 24, 36h post GVBD) at 37^{o}C in 5% CO_2 and 6% O_2, until further analysis.

In vivo matured (IVO) oocytes at the MII stage were obtained by priming mice with 7.5 IU/ml PMSG followed by 7.5 IU/ml human chorionic gonadotrophin (hCG, Chorulon®, Intervet, Boxmeer, The Netherlands) 48 hours later. Cumulus-enclosed oocytes were recovered from the ampullae 14 hours post-hCG and were released from the cumulus cells by a short incubation in 200 IU/ml hyaluronidase (0.3 mg/ml, type VIII) at 37^{o}C. Oocytes were washed intensively and transferred to microdrops of KSOM supplemented with 0.4% BSA (Lawitts and Biggers 1991). For *in vitro* ageing, IVO MII oocytes were incubated in this medium for eather 16 (fresh control), 24, or 36 hours post hCG (i.e. 2, 10 or 22 hours *in vitro* culture) at 37^{o}C under 5% CO_2 and 6% O_2 until further use.

Intracellular Ca^{2+} measurements

For measuring the Sr^{2+}-induced Ca^{2+}-oscillations, oocytes were loaded with 7.5µM of the Ca^{2+}-sensitive dye Fura-2 acetoxymethyl (AM) ester (Invitrogen, Life Technologies Europe B.V., Belgium). Following the loading, cells were washed intensively in Ca^{2+}-free KSOM medium and then transferred to a drop of parthenogenetic activation medium with 10 mM Sr^{2+} and 2µg/mL cytochalasin D (CCD) covered by light paraffin oil in the glass bottom dish (MatTek Corporation, Ashland, USA). Ca^{2+}-imaging was performed on an inverted epi-fluorescence microscope (TH4-200, Olympus Soft Imaging Solutions GmBH, Belgium) with a 20x objective. The microscope was equipped with an Okolab stage micro-environmental chamber enclosed in a CO_2 microscope cage incubator so that all measurements were conducted at 37°C under 6% CO_2. Fluorescence was recorded at an emission wave length of ~ 510 nm every 5 seconds for 2 hours with a filter switch that provided excitation alternating between 340 and 380 nm. Baseline drifting was adjusted before retrieving values for amplitude (value at maximum increase in fluorescence intensity per peak) expressed in arbitrary units (AU). Ca^{2+}-peaks were compared for latency period (the time between the exposure and the first Ca^{2+} rise, in hours) and frequency, which reflected the total number of oscillations per recording period.

To analyze their embryonic development potential, IVO and IVM MII oocytes were aged similarly and then parthenogenetically activated in Ca^{2+}-free KSOM containing $SrCl_2$ and CCD for 4 hours. The MII oocytes were washed and cultured in KSOM for the first 60-72 hours post-activation and embryos were then transferred to Cook Blastocyst medium (Cook; Sydney IVF Blastocyst medium, K-SIBM-50). Embryo development was assessed at 24 hours (two-cell), 72 hours (morula/early blastocyst) and 96 hours (blastocyst) post-activation.

Statistical analysis

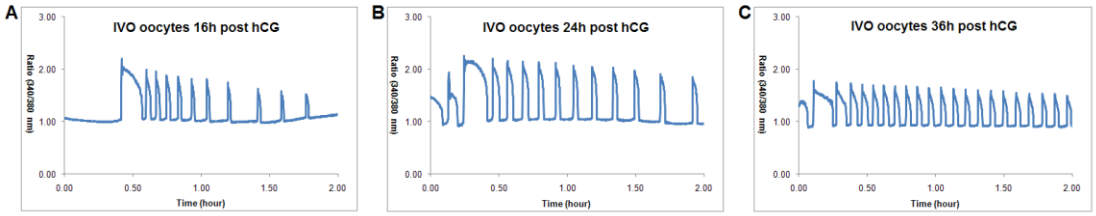
The Statistical Package for Social Sciences version 21 (SPSS® Statistics, IBM corp., NY, USA) was used for statistical analysis. Means (the frequency of the peak and RA) and Medians (the latency time period) of multiple groups were compared using ANOVA and Kruskal-Wallis test respectively. Proportions (Survival, activation and blastocyst formation rate) were compared by a contingency table analysis followed by a chi-square or Fisher's exact test. Differences yielding a *P* value <0.05 were considered as been statistically significant.

Results

Sr^{2+}-induced Ca^{2+}-oscillations after parthenogenetic activation of IVO and IVM post-ovulatory aged oocytes

Following their exposure to Sr^{2+}, the intracellular Ca^{2+}-levels of the oocytes promptly started to increase and showed a series of Ca^{2+}-oscillations. The first Ca^{2+}-peak of fresh oocytes is normally characterized by a rapid upstroke followed by a big sustained 'shoulder' and a slower decline towards the baseline (Figure 1). The repetitive Ca^{2+}-spikes of fresh oocytes were analyzed within a time interval of 2 hours and were compared to 24 hours and 36 hours post-hCG or post GVBD aged oocytes.

Figure 1. The Sr^{2+}-induced Ca^{2+}-rises of IVO fresh and post-hCG aged mouse oocytes during parthenogenetic activation.



Interestingly, 24 and 36 hours post-hCG aged IVO oocytes exhibited several small Ca^{2+}-rises before the first great peak (Figure 1), however, the latency period did not differ across the two post-ovulatory ageing groups and the fresh controls (media 0.01 vs. 0.01 vs. 0.01 hour). The percentage of survived oocytes that responded to Sr^{2+} by showing Ca^{2+}-oscillations was similar post parthenogenetic activation in the three IVO aged groups (98% vs. 100% vs. 87%) (Table 1). The frequency of the Ca^{2+}-rises was significantly increased in the two IVO aged

groups compared to the fresh IVO controls (mean 14.33 vs. 16.92 vs. 11.30) (P<0.05, Table 1). Furthermore, the RA of the IVO oocytes was increased in the 24 hours post-hCG aged IVO group compared to the fresh controls, followed by a significant decrease along with the time of ageing (36 hours post-hCG aged IVO oocytes) (Table 1).

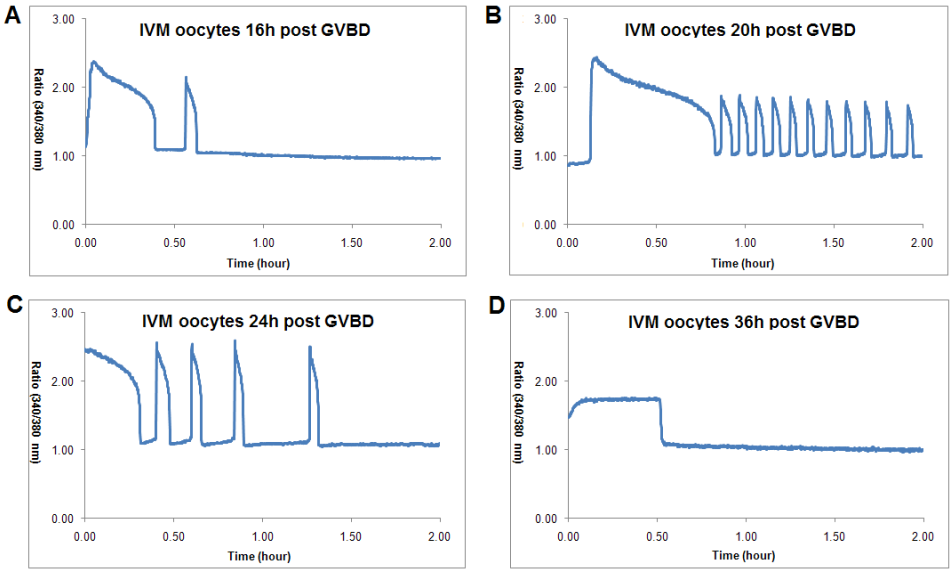
Table 1. Sr^{2+}-induced Ca^{2+}-oscillations during parthenogenetic activation in both IVO and IVM post-ovulary ageing oocytes.

Oocytes	Culture time post hCG/GVBD	No.	% oocytes Survived after Sr^{2+}	% Oocytes showing oscillations	Frequency of the Ca^{2+}-spikes	RA of the Ca^{2+}-spikes
IVO	16h	45	98%	98%	11.30±5.01[a,b]	0.89±0.22[a,b]
	24h	40	100%	100%	14.33±2.42	0.97±0.27[b]
	36h	30	87%	92%	16.92±8.26	0.67±0.15
IVM	16h	49	43%	78%	1.05±1.07[c,d]	1.00±0.63[c]
	20h	44	55%	92%	8.13±5.17	0.92±0.20[d]
	24h	33	42%	100%	6.57±2.79	1.11±0.30

ANOVA with Bonferroni's Multiple Comparison Test; [a]P<0.01 when compared with 24 hours post-hCG IVO oocytes. [b]P<0.01 when compared with 36 hours post- hCG IVO oocytes. [c]P<0.01 when compared with 24 hours post-GVBD IVM oocytes. [d]P<0.01 when compared with 36 hours post-GVBD IVM oocytes. Frequency and RA of Ca^{2+}-spikes are shown as Mean±S.D.

Upon the exposure of 36 hours post-GVBD aged oocytes (n=24) to Sr^{2+}, all oocytes were damaged. Therefore, we included the 20 hours post-GVBD group, to investigate the impact of ageing on IVM oocytes in depth (Figure 2). Similar to IVO oocytes, no difference was observed in the time period of lactency across all three IVM aged oocytes (media 0.04 vs. 0.03 vs. 0.02). Although the percentage of survived oocytes that showed Ca^{2+}-oscillations was similar among the three IVM aged groups (78% vs. 92% vs. 100%), significantly less oocytes survived from IVM groups (43% vs. 55% vs. 42%) during parthenogenetic activation, when compared to IVO aged groups (98% vs. 100% vs. 87%) (P<0.01, Table 1). Additionally, a significantly higher frequency of Ca^{2+}-oscillations was observed in 20 and 24 hours post-GVBD aged oocytes when compared to fresh IVM controls (P<0.01) (Table 1). All IVM groups showed a significantly lower number of Ca^{2+}-oscillations compared to IVO groups (P<0.01), except for the IVM 20 hours post GVBD group, showing a similar frequency of Ca^{2+}-oscillations as observed in IVO fresh oocytes (Table 1). Moreover, the RA of the Ca^{2+}-oscillations was decreased in 20 hours post GVBD aged IVM oocytes compared to fresh IVM oocytes, which was increased again in 24 hours post GVBD aged IVM oocytes when compared to 20 hours aged IVM oocytes (P<0.01) (Table 1).

Figure 2. The Sr^{2+}-induced Ca^{2+}-rises of IVM fresh and/or post GVBD aged mouse oocytes during parthenogenetic activation.



Embryonic development potential after parthenogenetic activation in both IVO and IVM post-ovulary aged oocytes

We compared the embryonic development potential of 24 hours and 36 hours post-hCG or post GVBD aged oocytes to fresh IVO or IVM MII oocytes after parthenogenetic activation. In the IVO groups, there was no significant difference in the percentage of oocytes that survived after parthenogenetic activation among all three groups. However, compared to the percentage (100%) of activated fresh IVO oocytes following Sr^{2+}-exposure after parthenogenetic activation, the 2-cell formation rate was significantly lower in 24 hours and 36 hours post-hCG aged oocytes (P<0.01, Table 2). Moreover, all oocytes with activation failure underwent fragmentation after 24 hours in these two aged groups. Nevertheless, there was no blastocyst formation in the 36 hours post-hCG aged group, while there was blastocyst formation in the 16 hours (80%) and 24 hours post-hCG aged oocytes (72%) (Table 2).

Table 2. Embryo development potential after parthenogenetic activation in post-ovulary ageing oocytes from both *in vitro* and *in vivo* maturation.

Ooocytes	Aged hours post hCG/GVBD	No.	Survival rate post activation (%)	2-cell formation rate (%)	Blastocyst formation rate (%)
IVO	16h	51	90%	100%[a,b]	80%[b]
	24h	52	100%	83%[b]	72%
	36h	30	93%	14%[a]	0%
IVM	16h	30	83%[d,e]	76%[e]	26%[c]
	20h	37	70%[e]	73%[e]	68%
	24h	35	51%[e]	56%[e]	60%
	36h	22	23%	0	N/A

Two-tailed Fisher's exact test; [a]*P*<0.01 when compared with 24 hours post-hCG IVO oocytes. [b]*P*<0.01 when compared with 36 hours post- hCG IVO oocytes. [c]*P*<0.01 when compared with 20 hours post-GVBD IVM oocytes. [d]*P*<0.01 when compared with 24 hours post-GVBD IVM oocytes. [e]*P*<0.01 when compared with 36 hours post-GVBD IVM oocytes. NA: not applicable

When subjecting IVM oocytes to Sr^{2+}, the survival rate was significantly lower in 24 hours (51%) and 36 hours (23%) post GVBD ageing groups compared to the fresh (16h) IVM group (83%) (Table 2). Following parthenogenetic activation, the two-cell formation rates were similar among 20 and 24 hours post GVBD aged oocytes and the fresh IVM controls, whereas the blastocyst formation rate was significantly higher in 20 hours post GVBD aged oocytes among all IVM groups (*P*<0.05, Table 2). Additionally, no differences were found in survival rate, or in 2-cell formation and blastocyst formation rate when compared 20 hours to 24 hours post GVBD aged oocytes (Table 2). Moreover, there was no 2-cell and blastocyst formation in 36 hours post-GVBD aged oocytes after parthenogenetic activation (Table 2). Furthermore, fresh IVM oocytes showed significantly lower blastocyst formation rates compared to fresh IVO MII oocytes (26% vs. 80%, Table 2).

Sr^{2+}-induced Ca^{2+}-oscillations during parthenogenetic activation in both IVM and IVO reproductive-aged oocytes

Reproductive ageing contributes to the increasing incidence of infertility, which is associated with a decrease in number and quality of ovulated oocytes. The Ca^{2+}-response of fresh IVO and IVM MII oocytes from 50-52 weeks old reproductive-aged mice were compared to fresh IVO and IVM MII oocytes from young mice during parthenogenetic activation in this study (Figure 3). The number of oocytes recovered from the aged mice was 5 times less than from the young mice, from both IVM and IVO (data not shown). Interestingly, the percentage of surviving oocytes (96% vs. 98%) and oocytes that showing Ca^{2+}-oscillations (100% vs. 98%) was similar in IVO oocytes from aged and young mice after parthenogenetic activation (Table

3). Moreover, no significant difference was found in the period of latency and the frequency of Ca^{2+}-oscillations, when compared IVO MII oocytes from old mice with IVO MII oocytes from young mice (Table 3). However, the RA of Ca^{2+}-oscillations of IVO MII oocytes from aged mice was significantly lower when compared to IVO MII oocytes from young mice (*P*<0.01, Table 3).

Figure 3. The Sr^{2+} induced Ca^{2+}-rises of IVO and IVM reproductive-aged mouse oocytes during parthenogenetic activation.

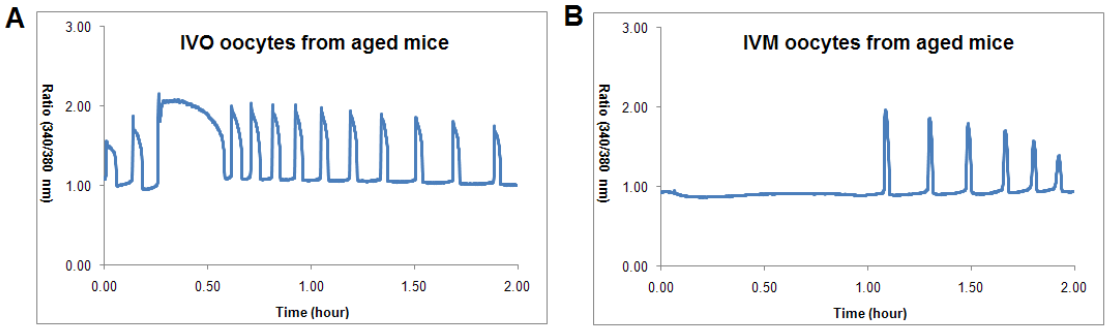


Table 3. Sr^{2+} induced Ca^{2+}-oscillations in both IVO and IVM oocytes from reproductive-aged mice.

	Oocytes (No.)	% Survived oocytes	% Oocytes showing Ca^{2+}-spikes	Latency (hour)	Frequency of the Ca^{2+}-spikes	RA of the Ca^{2+}-spikes
IVO	Aged (23)	96%	100%	0.20 (0.01-0.04)	10.4 ± 2.1	0.79±0.28[b]
	Young (45)	98%	98%	0.02 (0.01-0.54)	11.30±5.01	0.89±0.22[a]
IVM	Aged (22)	45%	90%	0.54 (0.21-1.49)	5.6 ± 3.1[a]	0.98±0.43
	Young (49)	43%	78%	0.04[b] (0.01-0.17)	1.05±1.07[b]	1.00±0.63

Student-t test and Mann-Whitney test. [a]*P*<0.01 when compared with IVO oocytes from aged mice. [b]*P*<0.01 when compared with IVM oocytes from aged mice.Frequency and RA of Ca^{2+}-spikes are shown as Mean±S.D. Latency is shown as median (range).

In IVM groups, no difference was found in the percentage of surviving oocytes (45% vs. 43%) and oocytes showing Ca^{2+}-oscillations (90% vs. 78%) when comparing aged mice versus young mice (Table 3). Interestingly, Sr^{2+} immediately initiated persistent oscillations in IVM oocytes from young mice. In contrast, there was a significantly longer latent period before the initial spike in the IVM oocytes from reproductive-aged oocytes, compared to IVM oocytes from young mice (media 0.54 vs. 0.04) (*P*<0.05, Table 3) (Figure 3). Furthermore, a

significantly higher frequency of the Ca^{2+}-oscillations with similar RA was found in IVM oocytes from aged mice compared to young controls (Table 3). The number of the Ca^{2+}-oscillations of IVM oocytes was significantly lower than IVO oocytes from aged mice, but displaying a higher RA (P<0.01, Table 3).

We further analyzed the embryonic development potential of oocytes from aged mice. No significantly differences were found in either activation rate or blastocyst formation rate, when compared IVM and IVO oocytes from aged mice to oocytes from young mice (Table 4).

Table 4. Embryo development potential after parthenogenetic activation in reproductive ageing oocytes from both *in vitro* and *in vivo* maturation.

	Origin of the oocytes	No.	Survival rate after activation (%)	2-cell formation rate (%)	Blastocyst formation rate (%)
IVO	Aged mice	35	97%	91%	84%
	Young mice	28	100%	96%	85%
IVM	Aged mice	22	72%	88%	71%
	Young mice	33	42%	100%	36%

DISCUSSION

Post-ovulatory and reproductive ageing have both been associated with a decreased fertilization capacity (Marston and Chang 1964; Ben-Rafael, Kopf et al. 1986; Badenas, Santalo et al. 1989), and increased abnormalities of the offspring (Tarin, Perez-Albala et al. 2001). However, the impact of ageing on the Ca^{2+}-oscillatory pattern of IVM and IVO oocytes had not been investigated yet. We found evidence of an abnormal Ca^{2+}-oscillatory pattern during parthenogenetic activation, which coincided with impaired embryo developmental potential in post-ovulatory aged oocytes, but not in reproductive-aged oocytes.

Intracellular Ca^{2+}-oscillations accompany all the metabolic and cytological changes at oocyte activation (Tombes, Simerly et al. 1992; Vasilev, Chun et al. 2012). Besides by PLCz, Ca^{2+}-rises can also be provoked by a variety of physical, mechanical and chemical activation stimuli (Vanden Meerschaut, Nikiforaki et al. 2014), of which Sr^{2+} is the most widely used artificial activating agent for rodent eggs (Whittingham and Siracusa 1978; Roh, Malakooti et al. 2003). However, the possible variation of Sr^{2+}-induced Ca^{2+}-pattern in different types of aged oocytes was still unknown.

In the current study, we found that the frequency of the Ca^{2+}-oscillations promptly increased in the 24 hours aged groups (10 hours *in vitro* culture), whereas, the RA of the Ca^{2+}-oscillations decreased in 36 hours post-hCG aged IVO oocytes. Prolonged culture ahead of activation

resulted in global changes in the abundance or activity of protein kinases that regulate the response to Ca^{2+}, stress, and cell-cycle control (McGinnis, Pelech et al. 2014). In addition to the kinase changes, the altered Ca^{2+}-oscillations of IVO post hCG aged oocytes can also be caused by decreased intracellular ER Ca^{2+} stores and impaired Ca^{2+} uptake by Ca^{2+}-ATPase (Funahashi, Cantley et al. 1994; Hao, Liu et al. 2009; Zhao, Liu et al. 2015), as well as down-regulated production of ATP content from mitochondrial oxidative phosphorylation (Day, Johnson et al. 1998). Most importantly, extended *in vitro* culture may influence the integrity of the actin cytoskeleton that affects the cycling of the transient receptor potential ion vanilloid 3 channels (Lee, Yoon et al. 2016), and thus alter the Sr^{2+}-induced Ca^{2+}-pattern in IVO post-ovulatory aged oocytes. These channels are recently defined as the mediators of Sr^{2+}-induced mouse oocyte activation (Carvacho, Lee et al. 2013). Moreover, the altered Ca^{2+} pattern is consisting with the studies in oocytes that were aged before fertilization (Takahashi, Takahashi et al. 2003; Takahashi, Igarashi et al. 2009).

Disrupted Ca^{2+}-homeostasis of *in vivo* aged oocytes is associated with the onset of apoptosis or programmed cell death upon fertilization (Gordo, Wu et al. 2000; Gordo, Kurokawa et al. 2002). Our result showed that adequate blastocyst formation rates could be obtained within 24 hours post-hCG/GVBD aged oocytes from both IVO and IVM groups. By extending the *in vitro* ageing procedure to 36 hours post-hCG/GVBD, a significantly higher percentage of degeneration was observed in both IVO and IVM groups, with 85% of IVO oocytes and 80% of IVM oocytes fragmenting/degenerating following activation. During *in vitro* ageing, the cytoplasmic factors that are crucial for successful embryogenesis (Schultz and Heyner 1992; Minami, Suzuki et al. 2007), genome remodelling (Torres-Padilla, Bannister et al. 2006), and DNA reparation (Genesca, Caballin et al. 1992) might be affected. The reduction of anti-apoptotic protein BCL2 during *in vitro* ageing might be contributing to the high percentage of oocyte fragmentation. (Takahashi et al. 2009).

Interestingly, the Ca^{2+}-pattern and embryonic development analysis demonstrated that IVM oocytes undergo more dramatic changes than IVO oocytes during *in vitro* ageing. We observed that an adequate embryonic development potential after parthenogenetic activation could only be obtained during a very narrow time window (20-24 hours post GVBD) after IVM, compared to fresh IVM oocytes. Moreover, all IVM groups exhibited significantly lower number of Ca^{2+}-oscillations compared to IVO aged oocytes. The observed compromised capacity of IVM MII oocytes to be activated and undergo development, may due to the reduced capacity of IVM oocytes to incorporate, release and use Ca^{2+} efficiently (Igarashi, Takahashi et al. 2005). Additionally, IVM human and mouse oocytes showed significantly lower concentrations of mtDNA and ATP content (Zeng, Ren et al. 2007; Ge, Tollner et al. 2012), as well as increased incidence of disrupted organization of the cytoskeleton and spindle configuration (Sanfins, Lee et al. 2003). Changes in the spindle position and distribution of IVM oocytes, in addition to their

inability to efficiently import Ca^{2+} to the site of injury, will result in cell death as well (Ouadid-Ahidouch 1998).

Oocytes from reproductive-aged individuals are associated with a decrease in number and quality of ovulated oocytes, and an increased percentage of abnormal or degenerating oocytes (Tarin, Perez-Albala et al. 2001), chromosomal aneuploidy (te Velde and Pearson 2002), and the injury of mitochondrial DNA (Keefe, Niven-Fairchild et al. 1995; Thouas, Trounson et al. 2005; Tatone 2008). The intracellular Ca^{2+}-oscillatory pattern was determined in 50-52 weeks old B6D2F1 mice in this study. Although the number of collected IVO MII oocytes was 5 times less from aged than young mice, we did not observe a higher number of abnormal or degenerated oocytes. The frequency of Ca^{2+}-oscillations exhibited by IVO oocytes was comparable in both groups. Interestingly, the frequency of Ca^{2+}-oscillations increased significantly in IVM oocytes from aged mice compared to young mice, with longer latency before showing the initial Ca^{2+}-rises after parthenogenetic activation. The lower metabolic state of IVM and aged oocytes may contribute to this result, as it is known that the dynamics of Ca^{2+}-oscillations are linked to the metabolic status of the oocyte (Lam and Galione 2013; Williams, Boyman et al. 2013). Upon low mitochondrial activation (FAD2+) and subsequent low mitochondrial ATP production via oxidative phosphorylation, the signal might be insufficient to start the initial Ca^{2+}-oscillation immediately after the parthenogenetic activation (Keefe, Niven-Fairchild et al. 1995; Dumollard, Marangos et al. 2004). The slow response could also result from a deficient enzymatic activity, which generates not enough ATP for Ca^{2+}-diffusing. IVM further aggravated the inability to incorporate Ca^{2+} signaling (Igarashi, Takahashi et al. 2005). Following the first Ca^{2+} rise, a rapid refilling of the Ca^{2+}-stores in the ER is observed, which is in accordance with the high frequency of Ca^{2+}-rises in oocytes (Wakai, Zhang et al. 2013). To verify the abnormalities of reproductive-aged oocytes, spindle-chromosomal alignment and aneuploidy, which are all closely related to ageing oocytes (Wakayama, Thuan et al. 2004; Mailhes 2008), further investigations are required.

We conclude that distinct deregulation of Ca^{2+} signaling is existing in post-ovulatory aged and reproductive-aged oocytes during oocyte activation. Moreover, the abnormal Ca^{2+}-pattern after parthenogenetic activation is related to the impaired embryonic developmental potential in post-ovulatory aged oocytes, but not in reproductive-aged oocytes. IVM oocytes showed an impaired Ca^{2+} oscillatory ability and subsequent embryo development potential, but only during a narrow time window following maturation to the MII stage. Further studies should be carried out to investigated the quality of the cytoskeletal structures as well as the Ca^{2+} transport in IVM oocytes.

Author's roles

Y.L., B.H. and P.D.S. conceived and designed the study. Y.L., M.B.F. performed experiments reported in Tables 1-3 and Figures 1-3. Y.L. B.H., J.N. and M.V.d.J. wrote the manuscript and assembled the figures. All authors contributed to the interpretation of the results and the editing of the manuscript.

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Conflict of interest

The authors declare no competing financial interests.

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Chapter 7

7. General Discussion

Oocyte maturation and activation is intrinsically linked to Ca^{2+} signaling. The precise pattern of Ca^{2+} oscillations are necessary for successful fertilization, but can exert long-term downstream effects on both gene expression and embryo development. From the experiments carried out in this thesis, we obtained a better understanding of the role of Ca^{2+} during oocyte maturation, activation and ageing. The deregulation of Ca^{2+} signaling during these three processes are shown to be associated with certain aspects of female related infertility.

7.1 Ca^{2+} signaling alterations during oocyte meiotic maturation

During IVM, oocyte maturation is accompanied by spontaneous Ca^{2+} fluctuations released through the IP_3R1 at GVBD and are crucial for meiotic progression and subsequent successful fertilization (Carroll and Swann, 1992). In chapter 3, we utilized the LT/Sv mouse strain to investigate Ca^{2+} signaling of IVM oocytes. The female LT/Sv mice show a high number of immature oocytes following ovulation induction, most of which remain arrested at the MI stage (Eppig, 1978; Hupalowska *et al.*, 2008). This meiotic arrest is very similar to those observed in human showing maturation resistant oocytes (Beall *et al.*, 2010). We further found impaired Ca^{2+}-signaling during IVM of LT/Sv GV oocytes, with lower frequencies and amplitudes.

Although it could be expected that the phenotypes or mutations from mouse maturation resistant oocytes can differ to those seen in humans, we observed very similar results regrading nuclear maturation of LT/Sv mouse oocytes compared to oocytes obtained from patients with >40% immature oocytes at retrieval. We could not perform Ca^{2+} analysis during IVM of human maturation resistant oocytes due to the lack of available oocytes in these patients. The observed Ca^{2+} signaling abnormalities may serve as a potential diagnostic method to investigate the cytoplasmic competence of IVM oocytes from maturation resistant patients. The role of abnormal Ca^{2+} signaling in human maturation resistance requires further validation.

Compared to the amount of freshly retrieved LT/Sv maturation resistant oocytes, we obtained a higher proportion of maturation resistant oocytes following the *in vitro* culture of LT/Sv oocytes from GV stage. Abnormalities in organelles and the cytoskeleton that may occur during IVM are possible causes for maturation resistance, fertilization failure and reduced developmental embryo development (Neri *et al.*, 2014). In mammals, oocytes undergo a prolonged and carefully regulated developmental processes, as a result of instructive paracrine and junctional interactions with their surrounding somatic cells. This dialogue between oocyte and somatic cells allows for the exchange of many regulatory signals that control oocyte metabolism, cytoskeletal remodeling, cell cycle progression and fertilization, all

of which are key events for initiating and sustaining early embryogenesis (Baudat *et al.*, 2013). In *vivo*, the immature GV stage oocyte maintains direct communication with the surrounding cumulus and granulosa cells through gap junctions, permitting heterologous metabolic and electrical coupling (Homa, 1995). Therefore, co-culture of the immature oocytes with cumulus cells and treatment with conventional IVF may be helpful for patients for which an abnormally high number of immature oocytes were retrieved. However, the clinical outcome of patients showing a high number of retrieved immature oocytes is largely unknown.

Interestingly, IVM oocytes retrieved from young mice showed restored Ca^{2+} signaling during prolonged (4-8h) *in vitro* culture in contrast to fresh IVM oocytes (16h post GVBD) (observed in Chapter 6). An adequate capacity to generate Ca^{2+} signals to support embryonic development after parthenogenetic activation could be obtained only in a narrow window (20-24 hours post GVBD) during the IVM process in mouse. The compromised capacity of IVM MII oocytes may be due to the reduced ability of incorporating and generating Ca^{2+} (Igarashi *et al.*, 2005) or due to declined concentrations of mitochondrial DNA and ATP content in IVM oocytes (Zeng *et al.*, 2007; Ge *et al.*, 2012). Furthermore, the increased incidence of abnormal cytoskeleton organization and aberrant spindle configuration during IVM may also be attributed to these deficiencies (Sanfins *et al.*, 2003).

Several studies have demonstrated that GV nuclear or cytoplasmic transfer represent potential treatment techniques to alleviate oocyte meiosis arrest, as shown in the maturation resistant mouse model (Hoffmann *et al.*, 2012) and to overcome defects in human oocyte maturation (Cohen *et al.*, 1997; Zhang *et al.*, 1999). Nuclear transfer has been successfully applied for the treatment of mitochondrial diseases in a mouse model (Neupane *et al.*, 2014) and more recently has been also applied in human to overcome mitochondrial diseases or even for infertility purposes such as embryonic arrest (Craven *et al.*, 2017). Therefore, nuclear transfer may serve as highly effective therapeutic approach for overcoming maturation resistance in the clinic. Certainly, in depth embryo developmental and safety studies are required prior to the clinical implementation of such treatments.

7.2 Ca^{2+} signaling alterations and Ca^{2+} based AOA treatments during fertilization

At fertilization, hyperactivated sperm release the sperm specific factor PLCζ. Downstream, PLCζ hydrolyzes PIP_2 to form IP_3 and thereby triggers Ca^{2+} release from the ER by binding to its receptor, IP_3R (Swann and Lai, 2013; Kashir *et al.*, 2014). The pattern of Ca^{2+} signaling at fertilization varies between species (Busa and Nuccitelli, 1985; Gillot and Whitaker, 1994), however, the total amount of intracellular Ca^{2+} must reach a crucial threshold for successful oocyte activation. Some human sperm is not able to activate human oocytes in *vitro* causing fertilization failure after ICSI. For example, globozoospermic sperm cells usually cannot activate mouse or human oocytes by the failure of inducing a normal Ca^{2+} oscillatory pattern

(Kashir *et al.*, 2010). More recently, more patients were identified whose sperm show aberrant calcium patterns after injection into mouse or human oocytes (Vanden Meerschaut *et al.*, 2014).

To diagnose sperm activation deficiency, mouse based assays are commonly used in patients experiencing ICSI failures (Rybouchkin *et al.*, 1996; Heindryckx *et al.*, 2005). The mouse oocyte activation test is an established diagnostic test performed in patients participating in our fertility program, prior to the application of AOA (Heindryckx *et al.*, 2005; Vanden Meerschaut *et al.*, 2012). It involves the injection of human sperm into mouse oocytes to determine sperm activation capacity (Rybouchkin *et al.*, 1996). In certain cases, the sensitivity of the mouse model can be further enhanced with the inclusion of Ca^{2+} analysis of both mouse and human oocytes. From our unpublished data, we demonstrate that oocyte Ca^{2+} analysis is a novel strategy for establishing human sperm activation deficiencies in patients experiencing ICSI failures. ICSI-AOA restores the fertilization rate only in patients displaying abnormal Ca^{2+} oscillations during human oocyte activation.

To date, the application of different AOA strategies to overcome fertilization failure after ICSI, has led to successful outcomes (Heindryckx *et al.*, 2005, 2008; Kyono *et al.*, 2009; Terada *et al.*, 2009; Ebner *et al.*, 2012; Kim *et al.*, 2014b). The commonly used AOA agent 'ionomycin' is a membrane permeable Ca^{2+} ionophore, which acts by creating pores in plasm membranes. The different Ca^{2+} transit pattern of oocytes from different species may be due to varying permeability to ionomycin or the distinct activity of the ER localized Ca^{2+} channels. Ionomycin will first act on the plasma membrane triggering Ca^{2+} entry; it will then diffuse into the oocyte where it may also affect the ER, where it can lead to an additionally release of Ca^{2+}. Alternatively, there may be Ca^{2+}-induced Ca^{2+} release. Calcimycin (A23187) acts similarly during oocyte activation, but is less potent and specific than ionomycin at the same concentration in various species (Kauffman *et al.*, 1980; Vasilev *et al.*, 2012; Nikiforaki *et al.*, 2016).

Currently, the non-standardization of protocols across laboratories and numerous variables affecting the procedure have also resulted in inconsistent data and reduced AOA efficiencies. In some instances the application of AOA protocols have in fact led to reduced fertilization and pregnancy rates, or a lack of improvement in outcomes when compared to routine ICSI (Borges Jr. *et al.*, 2009a, 2009b; Ebner *et al.*, 2012; Montag *et al.*, 2012). In Chapter 4, we showed that the use of suboptimal conditions during the application of AOA during a short time interval of ten minutes, had major effects on the subsequent oocyte activation rate and more importantly severely hampered subsequent embryonic development. The in *vitro* culture of the mouse oocytes exposed to different AOA conditions was performed in the same culture system (KSOM/Cook Blastocyst) in all the experimental groups. Therefore, the observed

effects on AOA can only be attributed to the varying conditions during this short timeframe of 10 minutes.

The availability of human oocytes for research is very low, the only sources of human oocytes for research are IVM oocytes or IVO oocytes with aggregates of SERs which are not clinically used in our center or failed fertilized oocytes after IVF/ICSI and cryopreserved oocytes, which cannot be used for the patient. Therefore, mouse models play an important role to elucidate the mechanism of Ca^{2+} signaling during the AOA processes.

In these experiments, mouse oocytes were utilized. We used KSOM medium, the most commonly used culture medium for mouse embryos. We designed 1xCa to 6xCa KSOM medium to evaluate the influence of external Ca^{2+} on the altered Ca^{2+}-transient pattern and subsequent embryonic developmental potential. In KSOM medium, all components are known, thus the observed changes should exclusively result from the altered concentrations of external Ca^{2+}, instead of the previously mentioned components supplemented in the culture media. However, KSOM is not the optimal culture medium for culturing human oocytes. Therefore, we kept the same culture media for activating and culturing the human oocytes, which are routinely used in human IVF labs.

We demonstrated that AOA efficiency is largely influenced by the external amount of Ca^{2+} and the type of AOA agent. Therefore, we encourage IVF laboratories to use more standardized protocols for AOA and account for the possible effects of parameters such as the culture media, and the type and concentration of the AOA chemicals used during the procedure. Moreover, the variability of other components amongst various culture media could also impact oocyte Ca^{2+} influx during activation (Morbeck *et al.*, 2014a, 2017). For example, variations in the composition of protein supplements and the concentration of Magnesium ions in the medium has been shown to influence blastocyst developmental potential in mouse (Graeff *et al.*, 1995; Morbeck *et al.*, 2014b). Further investigations are required to verify the effect of all these components on Ca^{2+} influx potential, and their subsequent effects on fertilization rates and embryonic developmental potential. Furthermore, AOA efficiency using ionomycin in combination with different commercial IVF media in human oocytes requires further investigation. Different combinations and concentrations of activating agents with certain types of commercial media could impose a different effect on the Ca^{2+} influx pattern.

Therefore, we recommend that fertility clinics applying AOA should take this into account. Currently, more standardized protocols that synchronize the crucial components used during AOA are warranted. Standardization and necessary diagnostic testing needs to be performed, for instance, for couples experiencing failed fertilization after ICSI. Both the mouse oocyte activation test and mouse oocyte Ca^{2+} analysis represent heterologous ICSI models to

distinguish sperm- from oocyte-related activation deficiencies (Rybouchkin *et al.*, 1996; Araki *et al.*, 2004; Heindryckx *et al.*, 2005). Moreover, Ca^{2+} oscillatory patterns can be an alternative method to evaluate the efficiency of different AOA methods.

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 PLCζ at fertilization. To date, Sr^{2+} has also been applied as an AOA method to overcome fertilization failure or low fertilization rates (Kyono *et al.*, 2008; Kim *et al.*, 2012, 2014a). To enable activation in mouse oocytes following Sr^{2+} exposure, extracellular Sr^{2+} permeates through its functional membrane channel, TRPV3 (Carvacho *et al.*, 2013), promoting downstream oscillations in $[Ca^{2+}]i/[Sr^{2+}]$ of the oocytes, likely by sensitizing IP_3Rs and thus facilitating Ca^{2+} oscillations (Zhang *et al.*, 2005), or substituting for Ca^{2+} in the potentiation of IP_3Rs (Girard and Clapham, 1993; Marshall and Taylor, 1994; Lee, 2016).

In Chapter 5, we demonstrated that Sr^{2+} was not capable of inducing any Ca^{2+} increase or provoking activation of human oocytes. This could be attributed to the inability of residues to conduct Sr^{2+} (Latorre *et al.*, 2007), as well as the insensitivity of IP_3R1 in responding to Sr^{2+} in human oocytes. However, Sr^{2+} application in clinics has overcome fertilization failure after ICSI, and consequently improved fertilization rates and embryo quality in couples with repetitive fertilization failure (Kyono *et al.*, 2008; Chen *et al.*, 2010), resulting in healthy live-births. The most likely explanation may be due to the lack of diagnostic methods used in such studies and thus the inability to show a true sperm-related activation deficiency in reports claiming to overcome failed fertilization by AOA with Sr^{2+} (Kyono *et al.*, 2008; Chen *et al.*, 2010; Kim *et al.*, 2014a).

Although the TRPV3 protein, expressed in human oocytes was shown to be functional in supporting agonists that triggered Ca^{2+} rise and oocyte activation in both mouse and human oocytes, oocytes failed to conduct Sr^{2+} influx or the conducted Sr^{2+} was not sufficient to promote Ca^{2+} release from the ER. In view of improving the efficiency of activating rodent oocytes, 2-APB was suggested as a potential AOA agent targeting the TRPV3 channels (Lee, 2016; Lee *et al.*, 2016). However, in our view, it is premature to encourage this at the moment, as both 2-APB and carvacrol activates multiple Ca^{2+} 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 Ca^{2+} influx originates exclusively from the external culture medium (Xu *et al.*, 2006; Lee *et al.*, 2016),

7.3 Ca^{2+} signaling deregulation and potential interventions in ageing oocytes

Oocyte ageing is the major determinant of developmental competence in embryos. The oocyte delivers half the chromosomal complement, but provides all the mitochondria DNA to the embryo. Therefore, maternal mitochondrial DNA may correlate to aging. Mice exhibit age-associated reproductive senescence in a similar manner as humans. Moreover, Ca^{2+}-signaling is deregulated in post-ovulatory aged oocytes at fertilization, showing higher frequency and lower amplitude of the released Ca^{2+} patterns (Kolker *et al.*, 2003; Takahashi *et al.*, 2009a). As such, instead of supporting normal embryonic development, these oocytes activate apoptosis, fragmentation, and cell death pathways (Gordo *et al.*, 2002; Takahashi *et al.*, 2009a).

During reproductive ageing, oocytes are exhibiting a decrease in number and quality of ovulated oocytes, with a concomitant increased percentage of abnormal or degenerating oocytes (Tarín *et al.*, 1999), chromosomal aneuploidy (te Velde and Pearson, n.d.), and injury in the mitochondrial DNA (Keefe *et al.*, 1995; Thouas *et al.*, 2005). Concordantly, we obtained a significantly lower number (five times less) of oocytes in 50-52 weeks old B6D2F1 mice compared to controls. The retrieved IVO oocytes exhibited a similar frequency of Ca^{2+}-oscillations as observed in young mice. However, the frequency of Ca^{2+}-oscillations increased significantly in IVM oocytes from aged mice compared to young mice, with longer latency before the initial Ca^{2+}-rises during parthenogenetic activation. Low mitochondrial activation (FAD2+) and subsequent low mitochondrial ATP production via oxidative phosphorylation may be insufficient to start the initial Ca^{2+}-oscillation immediately after parthenogenetic activation (Keefe *et al.*, 1995; Dumollard *et al.*, 2004). The lack of enzyme activity to produce ATP could therefore impair Ca^{2+} flux. Interestingly, once the Ca^{2+} oscillatory ability has been restored in IVM oocytes from aged mice, rapid refilling of Ca^{2+}-stores occurs, as observed in fresh young oocytes and in accordance with the observed high frequency of Ca^{2+}-rises in reproductive-aged oocytes (Wakai *et al.*, 2013).

Impaired Ca^{2+} signaling did not lead to defective embryo development in reproductive-aged oocytes. An adequate embryo development rate was observed in these oocytes following parthenogenetic activation, compared to controls. Mouse oocytes may be more tolerant to ageing-related errors than their human counterparts. It has also been reported that inhibiting or stimulating the natural pattern of Ca^{2+}-signaling of inseminated eggs has 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). Therefore, more human samples need to be investigated in order to understand the phenotypic variation and physiological consequences of reproductive and post-ovulatory ageing. Polar body genetic screening may provide a window into the oocyte's genome and telomeres, which might improve prediction of the developmental competence of the embryo (Keefe *et al.*, 2015).

Oocyte-transmitted mitochondrial DNA is known to be especially susceptible to aging. Therefore, we used low oxygen tension (5%) for the *in vitro* culture of oocytes and embryos. Exposure to high oxygen concentration may lead to high ROS generation and compromises embryonic developmental activity (Bavister, 2004). In the *in vitro* aged groups, we found increased frequency and decreased RA in post-ovulatory aged IVO oocytes, as reported previously (Takahashi *et al.*, 2009b). Prolonged culture prior to activation resulted in global changes in the abundance and activity of protein kinases that regulate the response to Ca^{2+}, stress, and cell-cycle control (MCGinnis *et al.*, 2014). In addition to the kinase changes, decreased $[Ca^{2+}]_{ER}$ and impaired Ca^{2+} uptake by SERCA (Funahashi *et al.*, 1994; Hao *et al.*, 2009; Zhao *et al.*, 2015), as well as down-regulated ATP production from mitochondrial oxidative phosphorylation (Day *et al.*, 1998), could also contribute to the observed Ca^{2+} signaling alterations. The Ca^{2+} transport during a Ca^{2+} rise requires the action of the SERCA, PMCA, as well as the Na^{+}/Ca^{2+} exchanger (Berridge *et al.*, 2000; Bootman *et al.*, 2001).

As we demonstrated distinctive Ca^{2+} patterns observed in two types of aged oocytes following parthenogenetic activation, cytoplasmic alterations may be different between reproductive-aged and post-ovulatory aged oocytes. Therefore, the etiologies and potential therapeutic approaches may also differ in these two types of aged oocytes. Reproductive ageing may occur as a result of inadequate ovarian angiogenesis in primordial follicles as well as in ovarian stroma vessels (Tatone, 2008). Hence, the activation of ovarian angiogenesis could be a new strategy for the improvement of the age-related decline of oocyte quality (Navot *et al.*, 1991). For post-ovulatory aged oocytes, caffeine treatment represents a potential therapeutic method. Applying 10mM caffeine to ageing bovine (Lee and Campbell, 2008), porcine and mouse oocytes (Iwamoto *et al.*, 2005), prevents the decline in MPF and MAPK activities and maintains normal meiotic spindle morphology (Ono *et al.*, 2011). Given the importance of Ca^{2+} in oocyte aging, further investigation is necessary, to develop therapeutic approaches for re-establishing normal Ca^{2+} signals in aged oocytes.

In summary, repetitive maturation resistance, failed fertilization and oocyte/maternal ageing represent certain types of infertility which are still challenging to overcome using currently available techniques (Bar-Ami *et al.*, 1994; Avrech *et al.*, 1997; Beall *et al.*, 2010; Heindryckx *et al.*, 2011). In this study, we demonstrated that these three processes are all associated with altered Ca^{2+} signaling. Our findings highlight plausible avenues for future diagnostic and therapeutic approaches in these types of human infertility.

Chapter 8

7. Future Perspectives

The significantly high percentage of nuclear abnormalities observed in oocytes obtained from aged patients and patients presenting with >40% immature oocytes at retrieval has hindered the clinical use of IVO and IVM oocytes as a potential treatment. Currently, no curative methods are available for such cases in the clinic. The prevalence of patients showing maturation resistance, fertilization failure and ageing is increasing, and therefore the need for accurate diagnostic and therapeutic approaches is becoming more prominent.

8.1 Ca^{2+} signaling analysis may be a potential diagnostic approach for ART failures

We performed Ca^{2+} analysis in maturation resistant and ageing oocytes. The observed defective Ca^{2+}-signaling of LT/Sv mouse maturation resistant oocytes at GVBD and during activation, sheds light on the physiological mechanism of human maturation resistant oocytes. This may allow for more effective diagnostic approaches in the future, such as the analysis of spontaneously released Ca^{2+} patterns in human immature oocytes during GVBD. We further establish variations in the Ca^{2+}-oscillatory pattern in reproductive-aged and post-ovulatory aged oocytes, which pointed to diverse cytoplasmic alterations. As such, our findings may guide the discovery of future therapeutic targets, specific for certain types of ageing oocytes. For instance, the IP_3R1 sensitizer may only be suitable in post-ovulatory aged oocytes, for restoring Ca^{2+} conductance. Furthermore, Ca^{2+} analysis may also be performed to evaluate the efficiency of potential therapeutic approaches. Correct Ca^{2+} signaling serves a crucial role in the completion of meiosis and initiation of embryo development. Therefore, re-establishing normal Ca^{2+} oscillatory patterns during meiosis maturation or oocyte activation may be a way to overcome maturation resistance and oocyte aging. In this regard, Ca^{2+} permeable channels ROC, TRPV and SOCE, SERCA, PMCA pathways may present potential targets.

To date, diagnostic methods such as the mouse oocyte activation test and mouse Ca^{2+} analysis have been effectively used to establish sperm-related oocyte activation failure (Vanden Meerschaut *et al.*, 2014). Following diagnosis, AOA methods are commonly applied to overcome fertilization failure. As the Ca^{2+} pattern of mouse oocytes may be influenced by external factors, such as components within the culture medium, evaluating the effect of such parameters on embryo development in mice may represents a suitable methodology for studying the influence of commercial culture media on human oocytes. However, as observed in our study, mouse oocytes are more sensitive to the effects of the surrounding environment compared to human oocytes; the value of mouse embryo tests to adequately reflect deficiencies in human oocytes requires further validation.

8.2 GV transfer represents a therapeutic approach for maturation resistant and ageing oocytes

In the limelight of growing concern over the cytoplasmic etiology of maturation resistance and oocyte ageing, nuclear transfer at the GV stage seems to be a possible therapeutic approach, currently with the most promise. Several attempts have been made to apply this form of nuclear transfer in maturation resistant oocytes from mouse models, with only one study succeeding in obtaining matured MII mouse oocytes following GV transfer (Hoffmann *et al.*, 2012). The authors reported that transferring the GV nucleus from LT/Sv oocytes into enucleated oocytes from OF1 wild-type mice resulted in 70% (24/33) of MI oocytes transiting to the MII stage (Hoffmann *et al.*, 2012). Furthermore, reconstructed xeno-oocytes containing a human GV nucleus and mouse cytoplasm obtained via GV transfer, demonstrated full maturation capacity (Zhang and Liu, 2015). This further demonstrates the possibility of re-establishing meiotic capacity by replacing the cytoplasm in maturation resistant oocytes.

Furthermore, in the field of human reproductive aging, GV transfer has been successful in preventing oocyte aneuploidy, commonly caused by ooplasmic dysfunction (Takeuchi *et al.*, 1999, 2001; Eichenlaub-Ritter *et al.*, 2004). By transferring GV nuclei of aged donors (>38 years) into the cytoplasm of enucleated oocytes of young donors (<31 years), 58% of the reconstructed oocytes completed meiosis, with 80% of these showing normal ploidy (Zhang *et al.*, 1999). Interestingly, Takeuchi *et al.* further demonstrated the potential capacity of reconstructed aged/young human oocytes to progress to the early cleavage stage of development (Takeuchi *et al.*, 2001). Our group also previously performed GV nuclear transfer (Neupane et al., 20??). However, the inability of the reconstructed mouse oocytes to develop into blastocysts following the intervention, limits the suitability of this approach and requires further optimization of the IVM process. Although highly abnormal spindle-chromosome configurations have been observed in both aged and maturation resistant oocytes in human patients and in the mouse model, the possibility of spindle transfer and PN transfer also requires further assessment. Moreover, further investigations into the underlying mechanisms providing the healthy cytoplasm the ability to correct spindle and nuclear abnormalities are also of great interest.

Nuclear transfer in both unfertilized oocytes and fertilized zygotes has shown promising results in mouse models, non-human primates and in humans (Craven *et al.*, 2017). Moreover, offspring born from non-human primates and mice after nuclear transfer has been shown to be normal and healthy (Craven *et al.*, 2017). Nuclear or cytoplasmic/mitochondrial transfer appear to be the most promising therapies in replacing the cytoplasmic origin of maturation resistance or minimizing the amount of aged mitochondrial DNA carryover. However, these techniques are still under investigation and require further validation in animal models before being applied for clinical trials in humans. Irrespective of the donor, ethical questions regarding

the involvement of the third set of (mitochondrial) DNA have also been raised and require further discussion. But currently, mitochondrial DNA copy number can be applied as a biomarker to identify high quality euploid embryos resulting from aged oocytes, with an aim to improve pregnancy outcomes.

8.3 Genetic analysis will likely benefit ART failures

Intensive knock-out studies in maturation resistance mouse models and gene screenings in human maturation resistant patients, have recently established seven mutations associated with MI arrest involving the candidate gene, TUBB8 (Feng *et al.*, 2016; Chen *et al.*, 2017; Huang *et al.*, 2017). This gene accounts for almost all expressed β-tubulin within the oocyte. The mutations affect α/β-tubulin heterodimer assembly and microtubule dynamics and thus result in spindle-assembly defects and maturation arrest, in human oocytes (Feng *et al.*, 2016; Chen *et al.*, 2017; Huang *et al.*, 2017). Therefore, specific gene modifications or mRNA interference are also a potential approach in diagnosing and treating such defects. Although promising, prior to applications in human, such interventions require comprehensive follow-up, both after fertilization and throughout the life of the offspring, to ensure their safety.

As part of this PhD research, we further described the difference between the Ca^{2+} oscillatory pattern in oocytes following reproductive aging and post-ovulatory aging. Aneuploidy *screening* by fluorescence *in situ* hybridization may reveal a deeper view of the ageing mechanism and provide possible therapies. In the clinic, several interventions that reduce oxidative stress and increase the proportion of euploid embryos have resulted in viable deliveries (Meldrum *et al.*, 2016). Newer interventions, such as CoQ10 or gene modification are emerging and promisingly improving age related dysfunctions (Meldrum *et al.*, 2016). Nevertheless, the benefits of these interventions on oocyte ageing require further investigation.

Exposure of oocytes to suboptimal culture conditions during the crucial activation phase (AOA), even during a very short time interval of 10min, not only has detrimental effects on oocyte activation, but also hampers downstream processes such as embryonic developmental potential. Here, we demonstrate the effects of external Ca^{2+} concentration, following the application of the most well-established AOA techniques. However, the impact of varied concentrations of other components in commercial human embryo culture media requires further investigation, examples include Mg^{2+}, Zn^{2+} and EDTA. Moreover, evaluating genetic profiles of oocytes or early stage embryos following different AOA protocols may aid in further establishing the effects of specific molecules during activation.

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List of Publications and conference contributions

Publications

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- **Y. Lu**, D. Bonte, M. Ferrer-Buitrago, J. Neupane, M. Popvic, M. Van der Jeught, L. Leybaert, P. De Sutter, B. Heindryckx. Culture conditions affect Ca^{2+} release in artificially activated mouse and human oocytes. Reproduction Fertility & Development 2017 (Accepted 2017).

- **Y. Lu**, R. Reddy, M. Ferrer Buitrago, J. Neupane, W.H. De Vos, E. Van den Abbeel, M. Vander Jeught, S. Lierman, P. De Sutter, B. Heindryckx. Strontium fails to induce Ca^{2+} rise and oocyte activation of human oocytes. Hum Reprod Open (Minor revisions).

- **Y. Lu**, M. Ferrer Buitrago, J. Neupane, , M. Vander Jeught, P. De Sutter, B. Heindryckx. In vitro culture effects calcium signaling of aged mouse oocytes during activation. Distinctions in Ca^{2+} oscillatory patterns of post-ovulatory aged and reproductive-aged oocytes following parthenogenetic activation. Manuscript in preparation 2017.

Contributions to other PhD research in G-FaST:

- Nikiforaki D, Vanden Meerschaut F, De Roo C, **Lu Y**, Ferrer-Buitrago M, De Sutter P, Heindryckx B. The effect of two assisted oocyte activation protocols used to overcome fertilization failure on the activation potential and calcium releasing pattern. Fertil Steril. 2016 Mar;105(3):798-806.

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- L. Liu, W. Huang, **Y. Lu**, A. Liao. Enhanced maternal anti-fetal immunity contributes to the severity of hypertensive disorder complicating pregnancy. Am J Reprod Immunol 2010; 63(5): 379-386.

Presentation in national/international conferences

- 31st annual meeting of European Society of Human Reproduction and Embryology (ESHRE); 14-17 June 2015, Lisbon, Portugal (POSTER)

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