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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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KEY MESSAGE

Patients with a high proportion of immature oocytes following routine ovarian stimulation, including at least one maturation resistant oocyte, fail to achieve pregnancy and their oocytes exhibit abnormal nuclear maturation. Appropriate treatment strategies, including clinical utilization of in-vitro maturation, require further investigation.

A B S T R A C T

Patients presenting with abnormally high numbers of immature oocytes at retrieval are more likely to exhibit maturation resistant oocytes. However, the clinical relevance of such events remains unknown. We investigated 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 or in-vitro matured (IVM). Treatment outcomes were evaluated in Study and Control groups based on 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 the Study group revealed significantly higher percentages of misaligned

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chromosomes compared with Controls ($P < 0.05$). Remarkably, Study group patients with at least one maturation resistant oocyte showed significantly reduced cumulative pregnancy and live birth rates compared with Control group maturation resistant patients ($P < 0.05$). When further investigating the aetiology, a maturation resistant mouse model revealed defective Ca^{2+} signalling of maturation resistant oocytes at germinal vesicular breakdown and parthenogenetic activation. In conclusion, appropriate treatment strategies, including clinical utilization of IVM oocytes from Study group patients, warrant further investigation.

Introduction

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 sub-fertile 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 (Beall et al., 2010; Bergere et al., 2001; Harrison et al., 2000; Heindryckx et al., 2011; Levran et al., 2002; Neal et al., 2002).

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 have a high probability of exhibiting maturation resistant oocytes, but the oocyte maturation competence and the pregnancy outcomes of these patients currently remain largely unknown. Both nuclear and cytoplasmic, structural or biochemical changes provide oocytes with the capacity to progress through meiosis (Fulka et al., 1998; Levran et al., 2002). Highly aberrant spindle-chromosome complex configurations have been observed in maturation resistant oocytes obtained from patients for which no mature oocytes were retrieved (Combelles et al., 2003; Heindryckx et al., 2011; Windt et al., 2001).

Moreover, evidence from a sub-fertile 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 to 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_3R) generating characteristic Ca^{2+} oscillations in oocytes, either released spontaneously or triggered by the sperm factor phospholipase C zeta (Ramadan et al., 2012; Saunders et al., 2002; Tesarik, 2002; Wakai et al., 2013). In LT/Sv mice, the Ca^{2+}-releasing ability and the nuclear maturation normality of collected fresh MI arrested oocytes remains unclear. Analysis of Ca^{2+} signalling 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 humans. Shedding light on the physiology of oocyte maturation in humans may allow for more effective diagnostic approaches.

In this study, the nuclear maturation competence of immature oocytes obtained from patients presenting with a high rate (>40%) of immature oocytes at retrieval (Study group), was compared 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 the Study and Control groups were classified for spindle-chromosome configuration analysis, based on their maturation status: maturation resistant or 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+} signalling 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 31 March 2009, 2010/182 approved on 21 April 2010 and 2010/808 approved on 20 January 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 12 December 2011.

Source of human oocytes

Patients (25–44 years old) undergoing intracytoplasmic sperm injection (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 administered a gonadotrophin-releasing hormone (GnRH) agonist (Decapeptyl; Ferring) or antagonist (Cetrotide; Merck Serono). Ovarian stimulation was performed by administering human menopausal gonadotrophin (HMG) (Menopur; Ferring) or recombinant FSH (Gonal-F; Merck Serono) at a dose of 112.5–300 IU daily and ovulation was induced with 5000 IU human chorionic gonadotrophin (HCG) (Pregnyl; MSD). Oocytes were enzymatically denuded by brief exposure to 80 IU/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 10 ng/ml epidermal growth factor, 1 mg/ml oestradiol, 10 mIU/ml recombinant FSH, 0.5 mIU/ml HCG, 1 mM L-glutamine, 0.3 mM sodium pyruvate, 0.8% (v/v) human serum albumin (Red Cross, Belgium), 100 IU/ml penicillin G and 100 mg/ml streptomycin sulphate at 37°C in 6% CO_2, 5% O_2. Based on first polar body extrusion, GV oocytes that reached the MII stage after 24 h or 48 h 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 reaching MII stage following 3 h or 24 h 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 h of culture were defined as GV-MI 48h. Donated MI oocytes which failed to extrude a first polar body within 24 h of in-vitro culture in Cook Cleavage medium were defined as MI-24h. For patients treated by ICSI, it was performed by standardized techniques in normal in-vivo matured MII oocytes. Fertilization rate (oocytes showing 2PN and a second polar body) was assessed 14–18 h post-injection.

Embryos were vitrified/warmed using a closed vitrification protocol (Irvine Scientific). Embryos were initially incubated in a 50 ml drop of Sydney IVF Gamete Buffer (GB) medium (Cook Ireland Ltd) for 1 min at 37°C and for 1 min at room temperature (RT). The GB drop was then merged for 2 min with an adjacent 50 ml 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 2 min before incubating the embryos in a separate 50 ml ES drop for 10 min at RT. Embryos were then sequentially incubated at RT in 4 drops of 25 ml vitrification solution containing 15% (v/v) ethylene glycol, 15% (v/v) DMSO and 0.5 M sucrose for 60–90 s in total. The embryos were finally loaded onto High Security straws (Cryo Bio System) with a minimal volume and the straws were thermosealed before being plunged into liquid nitrogen. For warming, embryos were immediately placed in a 500 ml drop of thawing solution containing 1 M sucrose for 1 min at 37°C and then for 1 min at RT. Embryos were subsequently placed in a 50 ml drop of thawing solution for 1 min at RT and then in two 50 ml drops of dilution solution containing 0.5 M sucrose for 2 min each. Finally, embryos were incubated in three 50 ml drops of washing solution for 3 min 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 endpoints 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. Miscarriage was defined as the spontaneous loss of a clinical pregnancy before 20 weeks of gestation 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 the 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 S1). Patients with 40% or more immature oocytes retrieved during an ICSI cycle represented 5% of the assisted reproductive population in our centre (based on the database from January 2003 to September 2011 in Ghent University Hospital). To define 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 (SER) from four patients (Supplementary Figure S1). The baseline characteristics of ICSI indication of the included patients are shown in Supplementary Table S1. No significant difference of the ICSI indication was found among all Study and Control groups.

Source of mouse oocytes

GV mouse oocytes were isolated from the ovaries of 8 to 14-week-old female LT/Sv (n = 25) and control B6D2F1 (n = 15) mice 44–48 h post-intraperitoneal injection of 7.5 IU/ml PMSG (Folligon®, Intervet) and were cultured in IVM medium for 16 h 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), 5 µg/ml insulin, 5 µg/ml transferrin, 5 ng/ml selenium (BD Bioscience), 50 mIU/ml FSH and 50 mIU/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 7.5 IU/ml PMSG, followed by 7.5 IU/ml HCG (Chorulon®, Intervet), 48 h later. Oocytes were recovered 14 h post-HCG. Cumulus cells were removed by short incubation in 200 IU/ml hyaluronidase. Potassium simplex–optimized medium (KSOM) was used for culture, while HEPES-buffered KSOM was used for manipulation. Both media were supplemented with 4 mg/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 IP3R1 staining

Both human and mouse oocytes were fixed in a microtubule-stabilizing buffer, as previously described (Heindryckx et al., 2011; Mattson and Albertini, 1990). Briefly, stripped in-vitro cultured or in-vivo matured oocytes were simultaneously fixed and extracted in a microtubule-stabilizing buffer (0.1 M PIPES, 5 mM $MgCl_2$, 2.5 mM EGTA, 0.01% aprotinin, 1 mM dithiothreitol, 50% deuterium oxide, 1 pM taxol, 0.1% Triton X-100 and 3% formalin) for 30 min at 37°C. Following three intensive washes (15 min each), oocytes were subsequently stored at 4°C in PBS-azide until fluorescence staining. At staining, oocytes were

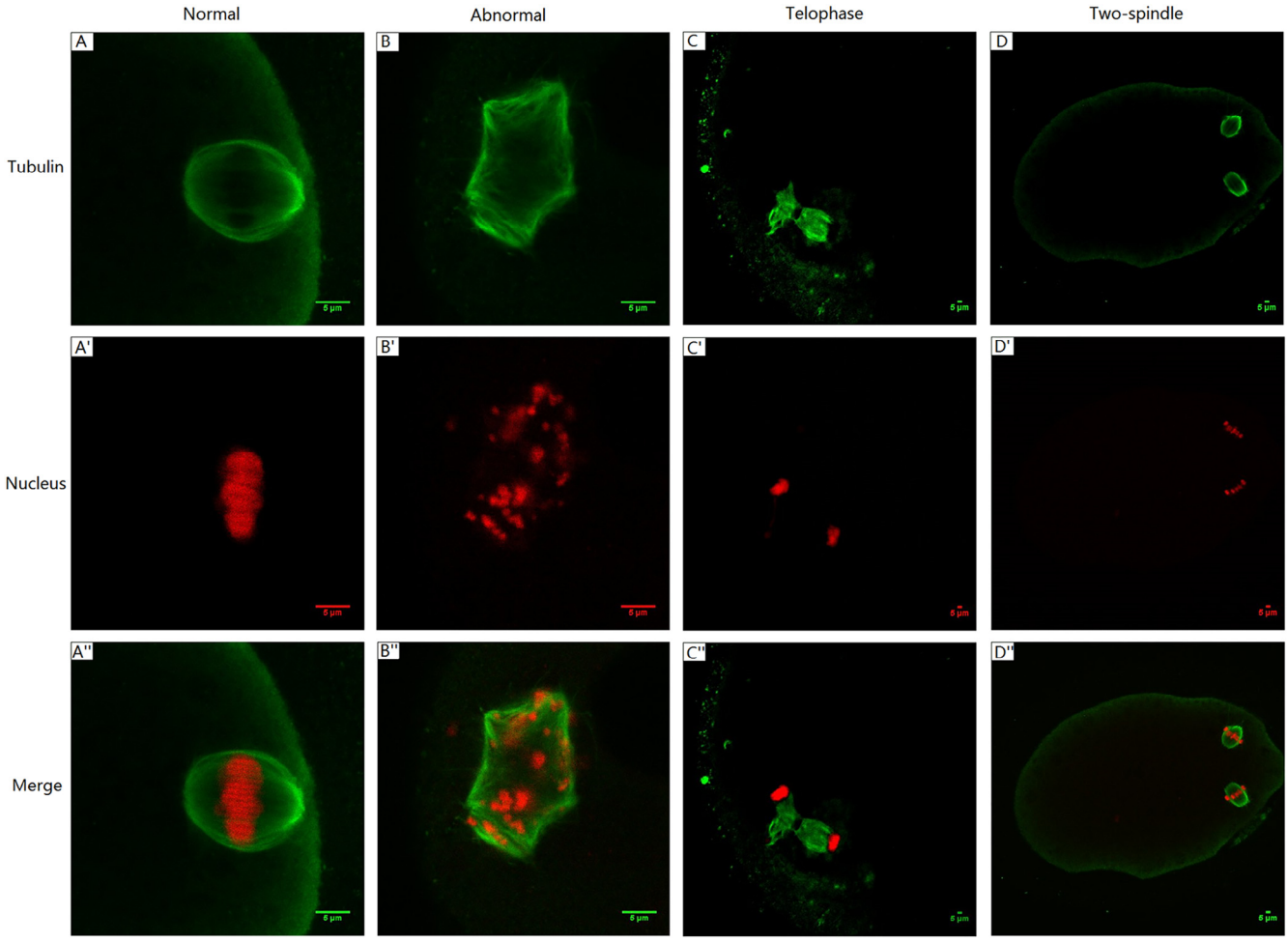

Figure 1 – Classification of spindle structure configuration and chromosome alignment in human oocytes. (A) Spindle with representative bipolar structure (normal). (B) Spindle with multipolar irregularities (abnormal). (C) Elongated telophase I spindle perpendicular to the oocyte membrane following 24-hour 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'' to D''). Scale bars: 5 μm.

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 (3 × 15 min), 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 1 h, followed by extended washing (3 × 15 min). The negative controls were treated with non-immune serum (1/1000) from the same rabbit before it was immunized for IP_3R1 (rabbit, KU Leuven). In addition, chromosomes were stained with Ethidium Homodimer-2 for 1 h for human and 20 μg/m Hoechst-33258 for 30 min 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 (0.5–0.75 μm/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 1A**), or abnormal (mono- or multipolar) (**Figure 1B**). The chromosomes were classified as aligned (**Figure 1A'**), or misaligned (>3 chromosomes away from the spindle equatorial region or dispersed with all chromosomes located throughout the spindle) (**Figure 1B'**) (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**), and no visible non-specific binding was observed in the non-immune serum negative controls, as suggested previously (Kim et al., 2011; Parys et al., 1995).

Intracellular Ca2 + measurements

Human oocytes were loaded with 7.5 μM of Ca^{2+}-sensitive dye Fura-2 acetoxymethyl (AM) ester (Invitrogen, Life Technologies) at 37°C in

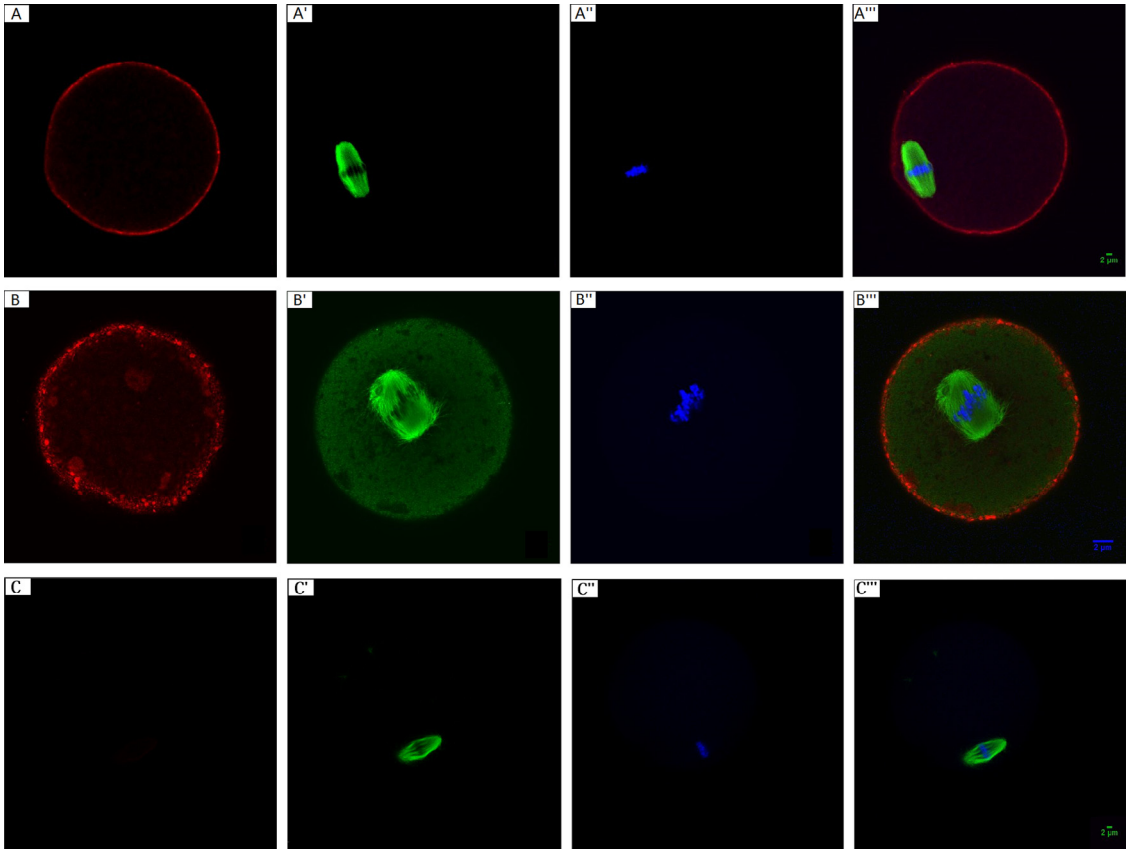

Figure 2 – Classification of IP_3R1 distribution in LT/Sv mouse oocytes. (A) Homogeneous IP_3R1 distribution with clusters on the cortex, forming a complete circle around the oocyte (Uniform). (A′) Normal spindle configuration and chromosome alignment (A′′) of B4D2F1 in-vivo matured MII oocytes. (B) Heterogeneous non-uniform distribution with patches and disconnection of IP_3R1 (Patched). (B′) Unfocused MI spindle poles and not perfectly aligned chromosomes (B′′) of LT/Sv mouse in-vivo collected maturation resistant oocyte. (C) Non-immune serum negative control with normal spindle configuration (C′) and chromosome alignment (C′′) of B4D2F1 in-vivo matured MII oocytes showing no IP_3R1 staining. (A′′′ to C′′′) Merged images of three confocal channels. Scale bars: 2 μm.

6% CO_2, 5% O_2 for 30 min 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 wavelength of ~510 nm every 5 s. The ratio of Ca^{2+}-induced signal (340 nm/380 nm) was proportional to the concentration of free intracellular Ca^{2+} (expressed in arbitrary units, AU).

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

Results

Nuclear maturation competence of human oocytes

In the Study group, oocytes were analysed 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 S1). 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 SER) were also included as controls (Supplementary Figure S1).

Spindle morphology was analysed by tubulin immunofluorescence (**Figure 1A–D**). Abnormal spindle configuration was observed in 62% and 60% of maturation resistant oocytes from the Study and Control groups, 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%

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 compared with Control in-vivo matured MII oocytes.

[b] $P < 0.05$ when compared with Control-IVM MII oocytes.

of the oocytes from the Controls, which was not significantly different between the two groups (**Table 1**). Interestingly, in the Controls, a similar percentage of oocytes with abnormal spindles was observed in IVM and in-vivo matured groups (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**).

The proportion of oocytes with misaligned chromosomes (**Figure 1B'**) was comparable in maturation resistant oocytes from the Study and Control groups (71% versus 64%, **Table 1**). Moreover, a significantly higher percentage of misaligned chromosomes persisted in the IVM MII oocytes from the 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 the 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, patients from the Study and Control groups were further divided into two subgroups: 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 patient's age or 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 transfer (fresh/cryopreserved) did not differ across all groups (**Table 2**). Moreover, no significant difference was observed in the median of the number of cryopreserved embryo transfers in current retrieval and the number of subsequent stimulations that has been performed (**Table 2**). 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**). Both pregnant patients from the Study-maturation resistant group ($n = 2$) suffered a miscarriage following the cryopreserved embryo transfer cycle in one case and a subsequent stimulation in the other case. However, no significant difference was observed in the miscarriage rate among all subgroups either from the current oocyte retrieval or across all cycles (**Table 2**). Of 11 patients allocated to the Study-maturation resistant group, four presented with all immature oocytes at retrieval, representing approximately half the number of analysed maturation resistant oocytes (16 out of 34). Conversely, from the Study-IVM patients, 38.5% achieved a clinical pregnancy, all of which resulted in 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, and the cumulative clinical pregnancy and live birth rate of Study-IVM patients were comparable to the Controls (**Table 2**).

In Control-maturation resistant patients, 32.6% achieved a clinical pregnancy, resulting in a live birth rate of 27.9% 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**). One ongoing pregnancy from the Control-IVM group following a subsequent stimulation was lost to follow-up, as the patient moved abroad. 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 SER were cancelled and no subsequent stimulation was performed. Another two such patients had a fresh embryo transfer and a subsequent stimulation, but no pregnancy was 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 IP3R1 distribution and Ca2 + oscillations during maturation and activation in LT/Sv maturation resistant oocytes

Based on the immunostaining pattern of spindle integrity (**Figure 1A'** and **1B'**), chromosome configurations (**Figure 1A''** and **1B''**) and IP_3R1 distribution (**Figure 2A–C**), 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 (both $P < 0.05$) and IVM MII oocytes from the B6D2F1 control

Table 2 – Embryological and cycle outcome data in the study and control patients for the investigated oocyte retrieval.[b]

	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 transfer (cryopreserved cycles)	1.2 ± 0.4	1.2 ± 0.4	1.1 ± 0.2	1.1 ± 0.4
Number of cryopreserved 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), *n* (%)	0	2/13 (15.4)	6/43 (14.0)	1/11 (9.1)
Clinical pregnancy plus cryopreserved embryo transfers, *n* (%)	1/11 (9.1)	5/13 (38.5)	14/43 (32.6)	3/11 (27.3)
Pregnancy across all cycles, *n* (%)	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, *n* (%)	0	0	2/18 (11.1)	0
Miscarriage for all cycles, *n* (%)	2/2 (100)	2/9 (22.2)	6/26 (23.1)	0
Live birth rate for fresh cycles, *n* (%)	0	2/13 (15.4)	6/43 (14.0)	1/11 (9.1)
Live birth rate in current oocyte retrieval, *n* (%)	0	5/13 (38.5)	12/43 (27.9)	3/11 (27.3)
Live birth rate for all cycles, *n* (%)	0[a–c]	7/13 (53.8)	20/43 (46.5)	6/11[d] (54.5)

ANOVA or Kruskal-Wallis or chi-squared test.
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 (%).
[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.
[d] The outcome of one pregnancy for a subsequent stimulation was unknown.

(both $P < 0.01$, **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**).

The maturation competence and the Ca^{2+}-releasing pattern of LT/Sv GV oocytes was investigated at germinal vesicle breakdown (GVBD), during in-vitro maturation. After 2 h 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**).

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

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 mature 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,b,c]	2 (11)	1 (5)	0
Misaligned chromosomes, N (%)	12 (63)	11 (61)	13 (76)[a,b,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.

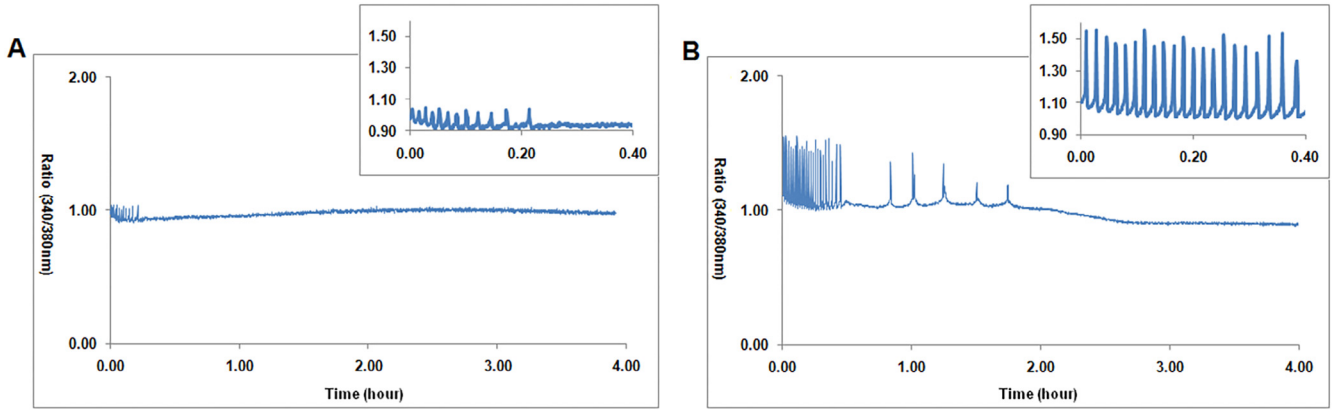

Figure 3 – Spontaneous Ca^{2+} oscillations during GVBD of LT/Sv and B6D2F1 oocytes. Ca^{2+} oscillations during GVBD of LT/Sv oocytes. (A) Ca^{2+} oscillations with a high-frequency amplitude of 0.3–0.7 at 1–3 min intervals during the first hour of measurement. (B) Ca^{2+} oscillations during GVBD of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

in-vivo matured LT/Sv MII and in-vivo matured B6D2F1 MII oocytes ($P < 0.05$, **Figure 4D–F**, **Table 4**).

Discussion

Although complete failure of oocytes to complete meiosis maturation during assisted conception cycles is rare, occasional and repetitive maturation resistance does occur in infertile patients (Avrech et al., 1997; Bar-Ami et al., 1994; Beall et al., 2010; Heindryckx et al., 2011). Patients presenting with an abnormally high number of immature oocytes at retrieval show a high probability of exhibiting maturation resistant oocytes, although the influence of such an 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+} signalling 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 births were 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 groups.

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 strontium exposure, N (%)	7 (30)[a]	18 (95)	24 (100)[c]	25 (100)	21 (43)[d]	45 (100)
Responded oocytes, 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] $P < 0.01$when 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.

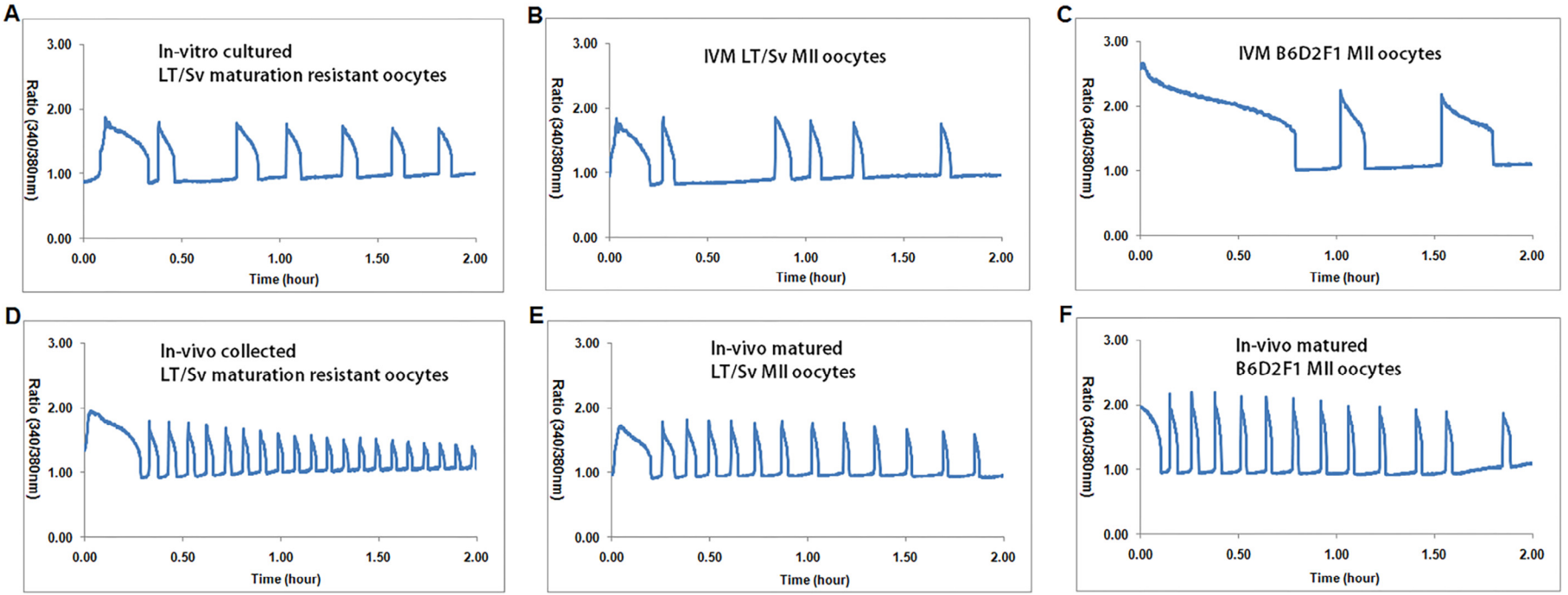

Figure 4 – The strontium-induced Ca^{2+}-release pattern in LT/Sv in-vitro cultured maturation resistant MI, IVM MII and B6D2F1 IVM MII control oocytes (A–C). The Ca^{2+}-release trace of LT/Sv in-vivo collected maturation resistant, in-vivo matured MII oocytes and B6D2F1 in-vivo matured MII oocytes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

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; Hoffmann et al., 2012; Maciejewska et al., 2009). 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-chromosome configurations, similar to observed human IVM oocytes from the Study group. We attempted to analyse 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+} signalling 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+} signalling 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 maturation resistant oocytes from our Study groups, the application of these methodologies requires further validation. Certainly, in-depth embryo developmental and safety studies are required before 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+} signalling at GVBD of in-vitro cultured LT/Sv maturation resistant oocytes and abnormal Ca^{2+} signalling 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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Appendix: Supplementary material

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