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Ca²⁺ dynamics in oocytes from naturally-aged mice

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The ability of human metaphase-II arrested eggs to activate following fertilisation declines with advancing maternal age. Egg activation is triggered by repetitive increases in intracellular Ca²⁺ concentration ([Ca²⁺]_i) in the ooplasm as a result of sperm-egg fusion. We therefore hypothesised that eggs from older females feature a reduced ability to mount appropriate Ca²⁺ responses at fertilisation. To test this hypothesis we performed the first examination of Ca²⁺ dynamics in eggs from young and naturally-aged mice. Strikingly, we find that Ca²⁺ stores and resting [Ca²⁺]_i are unchanged with age. Although eggs from aged mice feature a reduced ability to replenish intracellular Ca²⁺ stores following depletion, this difference had no effect on the duration, number, or amplitude of Ca²⁺ oscillations following intracytoplasmic sperm injection or expression of phospholipase C zeta. In contrast, we describe a substantial reduction in the frequency and duration of oscillations in aged eggs upon parthenogenetic activation with SrCl₂. We conclude that the ability to mount and respond to an appropriate Ca²⁺ signal at fertilisation is largely unchanged by advancing maternal age, but subtle changes in Ca²⁺ handling occur that may have more substantial impacts upon commonly used means of parthenogenetic activation.

Oocyte aging is a complex multifactorial process resulting in deterioration of oocyte viability with advancing maternal age. Perhaps the best known aspect of oocyte aging is oocyte aneuploidy, in which an age-related increase in chromosome segregation errors during meiosis is associated with a decline in female fertility^{1–3}. However, given their extraordinary protracted meiotic arrest, up to 45 years in humans, it is not surprising that oocytes are susceptible to other cellular dysfunctions with age. For example, clinical reports reveal that the ability of the oocyte to resume meiosis and begin embryo development following insemination⁴ or routine intracytoplasmic sperm injection (ICSI)^{5,6} also declines with advancing maternal age. Why oocytes from older women are less likely to respond appropriately to sperm is unknown.

At the time of ovulation mammalian oocytes become arrested at metaphase-II (MII) of meiosis, at which point the oocyte can be referred to as an egg. In mammals, liberation from MII arrest and initiation of the embryonic developmental program, commonly termed egg activation, occurs at fertilisation as a result of a spatiotemporal series of increases in intracellular Ca²⁺ concentration ([Ca²⁺]_i) within the egg cytoplasm known as Ca²⁺ oscillations. Ca²⁺ oscillations are initiated by a sperm-borne soluble protein, most probably phospholipase C zeta (PLCζ)⁷, and persist for several hours until the time of pronucleus formation⁸. Each Ca²⁺ oscillation is generated by inositol 1,4,5-trisphosphate (InsP₃)-mediated Ca²⁺ release from the endoplasmic reticulum (ER), the main intracellular Ca²⁺ store in the egg, followed by an influx of extracellular Ca²⁺ to replenish stores in time for the next oscillation⁹. Ca²⁺ oscillations are not only necessary and sufficient for egg activation^{10,11}, but their temporal dynamics also influence the developmental potential of the resulting embryo¹². Importantly, in clinical settings when egg activation fails following ICSI, eggs can sometimes be artificially activated by procedures that promote Ca²⁺ entry into the egg¹³. Yet, whether failed egg activation with advancing maternal age is caused by dysregulation of egg Ca²⁺ dynamics is not known.

A growing body of evidence, largely from naturally-aged mice, reveal changes in Ca²⁺ handling in various somatic cell types with age. This so-called calcium-hypothesis of aging is considered a major mechanism of age-related somatic cell dysfunction, and has particularly been studied with respect to the pathological pathways underlying Alzheimer's disease^{14–16}. Here we hypothesised that mammalian eggs might be similarly vulnerable

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to Ca^{2+} dysregulation with advancing maternal age, and that this would provide a mechanistic explanation for the reduced ability of eggs from older women to resume development after insemination or ICSI. Although some studies show that the latency period between ovulation and fertilisation either *in vivo* or *in vitro*, sometimes referred to as “post-ovulatory aging”, perturbs Ca^{2+} oscillations at fertilisation^{17–19}, whether maternal age affects the egg Ca^{2+} response is unknown. Therefore, using live fluorescence imaging, we performed the first examination of Ca^{2+} dynamics in eggs from young and naturally-aged mice. Perhaps surprisingly we find that, in contrast to somatic cells, $[\text{Ca}^{2+}]_i$ homeostasis remains relatively stable with advancing age, with naturally-aged eggs capable of mounting and responding to an appropriate Ca^{2+} signal at fertilisation. Instead, our experiments suggest mammalian eggs are adapted to avoid age-related Ca^{2+} signalling defects that might jeopardise reproductive capacity.

Results

Resting $[\text{Ca}^{2+}]_i$ and thapsigargin-sensitive stores are unchanged with age. Naturally-aged mice are an established model of *in vivo* aging, and have been extensively used to identify age-related changes in numerous cell types, including eggs. Here we used CD1 mice at 12–15 months old, which have been well characterised as a model for maternal age-related egg defects, as measured by aneuploidy levels^{20–23}. Control MII eggs from young CD1 mice and MII eggs from naturally-aged CD1 mice were collected contemporaneously 14 hours after hCG administration. We found that egg yields decreased markedly with age from 19.4 ± 1.2 per mouse in young mice to 2.8 ± 0.4 in aged mice ($P < 0.0001$) (Fig. 1a). These numbers are consistent with reports in the same mouse strain²⁰, and follow a similar rate of decline in the human ovary²⁴. Eggs from young and naturally-aged mice were morphologically indistinguishable (Fig. 1b). Specifically, young and aged eggs were identical in size, with mean diameters of $75.2 \pm 0.4 \mu\text{m}$ and $75.5 \pm 0.3 \mu\text{m}$ ($P = 0.54$) respectively, featured clear round zonae pellucidae, easily observable first polar bodies contained within perivitelline spacing of comparable size, and showed no obvious differences in egg shape or levels of cytoplasmic granularity (Fig. 1b), which is in line with morphological reports on ovulated human eggs of advanced maternal age²⁵. It is important to note that we also observed a small number of eggs from both young and aged mice that lacked cumulus cells at the time of collection, and were reduced in size and darker in appearance. These are commonly observed following a standard superovulation procedure and are presumed to be a result of an earlier ovulation, and therefore were not used in this study.

We first set out to determine if advanced age affects the ability to maintain appropriate cytosolic $[\text{Ca}^{2+}]_i$. MII eggs from young and naturally-aged mice were collected, microinjected with Calcium GreenTM-1 dextran and Rhodamine B dextran as a ratiometric imaging system, and imaged contemporaneously in a side-by-side manner using time-lapse epifluorescence microscopy in normal Ca^{2+} -containing media (Fig. 1b). The baseline mean fluorescence ratios of young and aged eggs were 0.97 ± 0.01 and 0.96 ± 0.02 ($P = 0.47$), respectively, revealing no significant difference in cytosolic $[\text{Ca}^{2+}]_i$ (Fig. 1c). Next we wanted to analyse the contents of intracellular Ca^{2+} stores. In pilot experiments we compared the Ca^{2+} response following treatment with ionomycin, a widely used Ca^{2+} ionophore, and found no difference in intracellular Ca^{2+} content between young and aged eggs (see Supplementary Fig. S1 online). Therefore, to determine more specifically whether ER Ca^{2+} stores are altered with age, we treated eggs with thapsigargin, a specific inhibitor of the ER Ca^{2+} -ATPase, that has been used extensively in oocytes^{26,27}. Thapsigargin causes a rise in $[\text{Ca}^{2+}]_i$ (Fig. 2a,b), which provides an estimate of the amount of Ca^{2+} contained within thapsigargin-sensitive ER stores in the egg ($[\text{Ca}^{2+}]_{\text{ER}}$). The calculated area under the curve for young and aged eggs was 252.8 ± 14.4 and 224.8 ± 16.1 ($P = 0.11$), respectively, and peak fold-change fluorescence levels of young and aged eggs were 1.69 ± 0.02 and 1.65 ± 0.03 ($P = 0.34$), respectively, revealing no significant difference in $[\text{Ca}^{2+}]_{\text{ER}}$ stores with egg age (Fig. 2c). Together these data show that cytosolic $[\text{Ca}^{2+}]_i$ and intracellular $[\text{Ca}^{2+}]_{\text{ER}}$ stores are maintained with age.

Naturally-aged eggs feature impaired Ca^{2+} influx. Ca^{2+} influx from the extracellular milieu is essential to sustain continuous Ca^{2+} oscillations from fertilisation until pronucleus formation^{28,29}. We therefore tested Ca^{2+} influx capacity in young and naturally-aged eggs. Following thapsigargin treatment in Ca^{2+} -free media, normal levels of extracellular Ca^{2+} were added back to the media (1.7 mM), which triggers an influx of Ca^{2+} into the egg and is an indication of store refilling (Fig. 2a,b). Strikingly, calculated area under the curve values decreased from 37.24 ± 5.02 in young eggs to 16.36 ± 3.46 in aged ($P = 0.0009$), and similarly peak fold-change fluorescence levels were reduced from 1.2 ± 0.02 to 1.1 ± 0.01 in young and aged eggs, respectively ($P = 0.0002$) (Fig. 2d). These data show that MII eggs from mice of advanced age possess a markedly reduced ability to replenish Ca^{2+} from the extracellular environment.

ICSI- and PLC ζ -induced Ca^{2+} oscillatory patterns are maintained with age. Normal sperm-initiated Ca^{2+} oscillations are dependent upon Ca^{2+} influx, and cease prematurely in the absence of external Ca^{2+} ^{30,31}. We therefore wondered whether the reduced Ca^{2+} influx capacity that we had detected in naturally-aged eggs would affect the dynamics of Ca^{2+} oscillations. Thus we performed ICSI on young and aged eggs and recorded $[\text{Ca}^{2+}]_i$ every 10 seconds for 3.5 hours using epifluorescence imaging. ICSI was chosen for this series of experiments to exclude the possibility of polyspermic fertilisation, which can occasionally occur with standard *in vitro* fertilisation, and could confound the results. As in all experiments young and aged eggs were imaged in a side-by-side contemporaneous manner. Ca^{2+} oscillations in all eggs persisted for the duration of imaging (Fig. 3a,b). Young and aged eggs displayed a similar number of Ca^{2+} oscillations, with 3.50 ± 0.23 and 3.30 ± 0.26 Ca^{2+} spikes ($P = 0.61$) in the first hour (Fig. 3c) and 5.64 ± 0.37 and 5.20 ± 0.47 Ca^{2+} spikes ($P = 0.46$) in the first two hours, respectively (Fig. 3d). Furthermore, peak increases in $[\text{Ca}^{2+}]_i$ between young and aged eggs were 2.19 ± 0.02 and 2.16 ± 0.02 ($P = 0.50$), respectively (Fig. 3e), suggesting that aged eggs are capable of

Figure 1. Cytosolic $[\text{Ca}^{2+}]_i$ concentrations are unchanged with maternal age. (a) Comparison of MII egg numbers collected from young and naturally-aged mice. Note the marked decrease in egg yields with advancing maternal age ($P < 0.0001$). Data collected from 20 independent experiments and presented as average number per mouse ($n = 87$ aged mice, $n = 37$ young mice). (b) *Top panel:* Young and aged eggs were imaged side-by-side for all experiments. *Bottom panel:* Representative ratiometric image of young and aged eggs injected with Calcium GreenTM-1 dextran and Rhodamine B dextran. (c) Quantification of cytosolic $[\text{Ca}^{2+}]_i$ in young ($n = 34$) and aged ($n = 22$) eggs (Ca^{2+} -containing media) revealed no difference with maternal age ($P = 0.47$). Data collected from 4 independent experiments. ns indicates not statistically significant. a.u. represents arbitrary units. Error bars are s.e.m.

Figure 2. Naturally-aged eggs feature a reduced Ca^{2+} influx capacity. (a) A typical Ca^{2+} response to thapsigargin followed by Ca^{2+} add-back is demonstrated with pseudocoloured time-lapse images, with warmer colours indicating higher $[\text{Ca}^{2+}]_i$. Numbers in the top left of each image panel represent minutes (mins). (b) Representative Ca^{2+} response curve in MII eggs from young and naturally-aged mice after treatment with thapsigargin ($10\mu\text{M}$) followed by Ca^{2+} (1.7mM) add-back. The Ca^{2+} add-back response curve has been magnified (grey box outline). (c) Quantitative analyses of the thapsigargin response in young ($n=18$) and aged ($n=10$) eggs. Area under the curve (AUC) and fold-change fluorescence ratio calculations revealed maternal age does not affect thapsigargin-sensitive $[\text{Ca}^{2+}]_{\text{ER}}$ stores ($P=0.11$ and $P=0.34$, respectively). (d) Quantitative analyses of the Ca^{2+} add-back response in young ($n=17$) and aged ($n=10$) eggs. AUC and fold-change fluorescence calculations showed Ca^{2+} influx capacity is reduced with maternal age ($P=0.0009$ and $P=0.0002$, respectively). Experiments performed in Ca^{2+} -free media. ns indicates not statistically significant. a.u. represents arbitrary units. Error bars are s.e.m.

mounting Ca^{2+} oscillations of appropriate amplitude. Together, these data show no significant difference in the ICSI-induced Ca^{2+} oscillation signature with advanced egg age.

To further examine Ca^{2+} oscillation patterns in naturally-aged eggs we set out to compare the effect of artificially introducing $\text{PLC}\zeta$, a physiological sperm-borne trigger of Ca^{2+} oscillations^{32,33}. $\text{PLC}\zeta$ was introduced by microinjecting cRNA encoding the $\text{PLC}\zeta$ protein tagged with firefly luciferase³⁴. Analogous to ICSI, $\text{PLC}\zeta$ -induced Ca^{2+} oscillations in all eggs continued for the duration of imaging (Fig. 4a,b). Young and aged

Figure 3. ICSI-induced $[Ca^{2+}]_i$ oscillations are unchanged with maternal age. (a,b) Typical Ca^{2+} oscillatory patterns in young and naturally-aged eggs following ICSI. (c,d) Quantification of the number of Ca^{2+} spikes in the first hour (c) and first two hours (d) in young ($n = 14$) and aged ($n = 10$) eggs revealed no difference in oscillation frequency ($P = 0.61$ first hour, $P = 0.46$ first two hours), indicating that aged eggs are capable of mounting and responding to an appropriate Ca^{2+} signal at fertilisation. (e) Quantification of fold-change fluorescence in young ($n = 14$) and aged ($n = 10$) eggs revealed the amplitude of Ca^{2+} oscillations is unaffected by age ($P = 0.50$). Experiments performed in Ca^{2+} -containing media. ns indicates not statistically significant. a.u. represents arbitrary units. Error bars are s.e.m.

eggs elicited 15.42 ± 1.31 and 14.58 ± 1.15 PLC ζ -induced Ca^{2+} spikes ($P = 0.97$) in the first two hours (Fig. 4c), respectively, revealing that like ICSI, the frequency of PLC ζ -induced Ca^{2+} oscillations are unchanged with egg age. Moreover, there was no significant difference in the amplitude of PLC ζ -induced Ca^{2+} oscillations between young and aged eggs, with fold-change increases in fluorescence ratio calculated as 3.86 ± 0.12 and 4.02 ± 0.23 ($P = 0.51$), respectively (Fig. 4d). To correlate oscillation number to PLC ζ protein expression, we performed a quantitative analysis of total luminescence on an egg-by-egg basis³⁴. Firstly, young and aged eggs featured similar PLC ζ protein expression levels with luminescence counts per second in the first hour of oscillations recorded as 2484.0 ± 116.9 and 2396.6 ± 195.2 ($P = 0.69$), respectively, indicating inherent translational efficiency is not compromised by age (Fig. 4e), consistent with expression of GFP-tagged fusion proteins by mRNA injection in young and aged germinal vesicle stage oocytes in our lab (JH and GF unpublished). Secondly, quadratic regression analysis of luciferase expression showed no relationship between oscillation frequency and protein expression across

Figure 4. PLC ζ -induced $[Ca^{2+}]_i$ oscillations are unchanged with maternal age. (a,b) Typical Ca^{2+} oscillatory pattern in young and naturally-aged eggs following microinjection with PLC ζ cRNA. (c) Quantification of the number of Ca^{2+} spikes in two hours following the first oscillation in young ($n = 31$) and aged ($n = 12$) eggs showed that maternal age does not affect the eggs ability to respond to a PLC ζ signal at fertilization ($P = 0.97$). (d) Quantification of the average oscillation amplitude in young ($n = 31$) and aged ($n = 12$) eggs showed no difference with age ($P = 0.51$). (e) Quadratic regression analysis of oscillation number and PLC ζ protein expression level (measured as luminescence levels) in young (black) and aged (red) eggs revealed age does not affect the sensitivity of PLC ζ -induced Ca^{2+} oscillations. R^2 values are 0.88 and 0.94 ($P = 0.56$) for young and aged eggs, respectively. Experiments performed in Ca^{2+} -containing media. F/F0 represents fluorescent intensity relative to baseline. cps represents counts per second. ns indicates not statistically significant. Error bars are s.e.m.

young and aged eggs, with R^2 values calculated at 0.88 and 0.94 ($P = 0.56$), respectively, indicating that age does not affect the sensitivity of Ca^{2+} oscillations initiated by PLC ζ expression (Fig. 4e). Collectively, our data show the ability to mount an appropriate ICSI- or PLC ζ -induced Ca^{2+} signal is unaffected by advancing maternal age.

Naturally-aged eggs exhibit substantially fewer oscillations in response to parthenogenetic activation by $SrCl_2$. Parthenogenetic agents are used in clinical settings to artificially activate eggs that do not spontaneously activate following ICSI³⁵, and are commonly used in an experimental context in many mammalian systems^{26,36}. We therefore examined oscillation competence in naturally-aged eggs following activation with $SrCl_2$, a parthenogenetic agent that is widely used in mouse and evokes repetitive oscillations similar

Figure 5. Naturally-aged eggs show a marked reduction in oscillation number following parthenogenetic activation with SrCl_2 . (a,b) Typical SrCl_2 -induced oscillatory patterns in young and naturally-aged eggs. (b) Note that two different oscillatory responses were observed for aged eggs. i) 61.5% of aged eggs oscillated for the duration of imaging (left panel). ii) 38.5% of aged eggs ceased oscillating prematurely (right panel). (c) Quantification of the number of oscillations in the first two hours in young ($n = 40$) and aged ($n = 13$) eggs revealed a marked reduction in oscillation frequency with maternal age ($P < 0.0001$). (d) Quantification of the fold-change fluorescence ratio in young ($n = 40$) and aged ($n = 13$) eggs revealed oscillation amplitude is unaffected by age ($P = 0.7$). Experiments performed in Ca^{2+} -free media. a.u. represents arbitrary units. ns indicates not statistically significant. Error bars are s.e.m.

to fertilisation^{37–39}. To do this, we incubated young and aged eggs side-by-side in SrCl_2 -containing media and recorded oscillations for 3.5 hours (Fig. 5a,b). SrCl_2 -induced oscillations were similar in amplitude in young and aged eggs ($P = 0.7$) (Fig. 5d). Notably however, unlike ICSI- and $\text{PLC}\zeta$ -induced oscillations, 38.5% of aged eggs prematurely ceased oscillating prior to the end of imaging (Fig. 5b), compared to only 7.5% of young eggs, showing that SrCl_2 -induced oscillation longevity is compromised with age. Overall, aged eggs displayed a 57% reduction in oscillation number compared to young, with 2.77 ± 0.41 oscillations in the first two hours compared to 6.45 ± 0.29 oscillations in young eggs ($P < 0.0001$) (Fig. 5c). Thus aged eggs feature a reduced ability to mount appropriate oscillatory responses following parthenogenetic activation with SrCl_2 .

It is known that the cellular events underpinning the egg-to-embryo transition are differentially regulated by a specific number of Ca^{2+} transients⁴⁰. Therefore to test whether the reduced longevity and frequency of Sr^{2+} -induced oscillations observed in aged eggs affected the temporal sequence of egg activation, we recorded the timings of second polar body (Pb2) extrusion and pronucleus (PN) formation in young and aged eggs, and correlated the timing of these events with total oscillation number on an individual egg-by-egg basis (Fig. 6a). Regardless of oscillation number, the timing of Pb2 extrusion and PN formation was similar across young and aged eggs (Fig. 6b,c). Together these findings show that advanced maternal age affects the ability of eggs to mount a normal oscillatory pattern in response to parthenogenetic activation with SrCl_2 , but this does not impact the temporal kinetics of egg activation.

Discussion

Egg activation failure is a prominent barrier to the success of ICSI in the clinic⁴¹, and is associated with advancing maternal age^{5,6}. Since the Ca^{2+} signal at fertilisation is both necessary and sufficient for egg activation, we hypothesised that eggs might be vulnerable to Ca^{2+} dysregulation with advancing maternal age. Although the effect of time after ovulation (often referred to as post-ovulatory aging) on egg Ca^{2+} regulation has previously been studied^{17–19}, the effect of maternal age was unknown. We took advantage of the recent characterisation of naturally-aged mice as a model of maternal egg aging and found that although Ca^{2+} influx capacity is markedly impaired, the ability to mount a normal sperm- and $\text{PLC}\zeta$ -induced Ca^{2+} oscillatory signature is largely unchanged. Interestingly, on the other hand, we find that eggs of advanced age elicit an abnormal oscillatory pattern in response to artificial activation with SrCl_2 . The discussion that follows will therefore first focus on the effect of aging on egg Ca^{2+} handling, and then comment on the effect of aging upon egg activation in a clinical context.

Whilst it is well established that oocyte aneuploidy is a major consequence of oocyte aging and a leading cause of age-related infertility^{1,42}, the prolonged lifespan of oocytes provides ample opportunity for other cellular

Figure 6. Egg activation kinetics are not affected by maternal age. (a) Timing of second polar body extrusion (Pb2) and pronucleus formation (PN) in young and aged eggs activated with SrCl_2 . Note that the timing of both egg activation events are similar, regardless of egg age. (b) Correlation between second polar body timing and oscillation number (total) between young and aged eggs. (c) Correlation between pronuclear formation timing and oscillation number (total) between young and aged eggs.

defects to develop. Indeed age-related changes in mitochondrial DNA arrangements⁴³, mitochondrial function^{44,45}, and gene expression profiles^{46,47} have been reported. Here we report that a maternal age-related deterioration in Ca^{2+} influx occurs in eggs. Reductions in store-operated Ca^{2+} entry (SOCE) activity and expression of key SOCE-specific proteins, Stim1 and Orai1, are a characteristic feature of naturally-aged mitotic cells^{48,49}, however their behaviour during egg aging is not known. We speculate that an age-related deterioration in SOCE channel expression or activity likely explains our observation of impaired influx in aged eggs following ER store depletion. Intriguingly however, our data show that Ca^{2+} oscillations remain robust and unchanged in eggs from older females in the face of substantially reduced store-operated Ca^{2+} influx. Moreover no obvious difference in the pacemaker potential was observed, though more detailed analysis of this aspect of the Ca^{2+} oscillation would benefit from more rapid sampling. Whilst fertilisation in nominally Ca^{2+} -free media results in oscillation termination³⁰, revealing that at least some extracellular Ca^{2+} is essential for ongoing long-term oscillations, our data suggest that Ca^{2+} oscillations can persist provided a threshold amount of Ca^{2+} influx is available. Consistent with this, SOCE inhibition at fertilisation has no effect on the Ca^{2+} oscillatory response²⁸, similar to the post-fertilisation Ca^{2+} oscillation phenotype of the aged eggs in this study. Nonetheless an unavoidable conclusion of our data is that, in contrast to other cell types in which an age-related dysregulation of Ca^{2+} homeostasis is common^{14,15}, in eggs the ability to generate Ca^{2+} transients is apparently safeguarded. This difference in the pathogenesis of aging between somatic cells and oocytes may be unique to Ca^{2+} , as aged oocytes feature other hallmarks of somatic cell aging, such as an accumulation of reactive oxygen species⁵⁰, consistent with the involvement of free radicals in aging⁵¹. Whilst detailed analyses of other aspects of ionic homeostasis with oocyte age, such as pH regulation⁵², remain to be studied, we speculate that oocytes are specifically protected from Ca^{2+} aging, as a mechanism of avoiding endangering the germline.

Whereas we found little effect of natural maternal aging upon sperm- or PLC ζ -induced Ca^{2+} oscillations, our data reveal that aged eggs exhibit a substantially different oscillatory response following activation with SrCl_2 compared to young. Perhaps surprisingly, given its widespread use in mouse eggs as a parthenogenetic agent, exactly how SrCl_2 initiates a sustained train of oscillations remains poorly understood. Having entered the egg, SrCl_2 is thought to stimulate oscillations by potentiating the InsP_3 receptors (InsP_3R)³⁹. Recent elegant studies identified that TRVP3, a specific transient receptor potential ion channel, is the major route of SrCl_2 entry into mouse oocytes, with $\text{TrpV3}^{-/-}$ eggs failing to mount an oscillatory response, presumably as a result of failed access to the InsP_3R ⁵³. Thus a simple potential explanation is that an age-related reduction in TRVP3 might be responsible both for the inhibition of SrCl_2 -induced oscillations, and for the subtle defects in Ca^{2+} influx. It is also plausible that defects in downstream signalling such as possible activation of oocyte-resident PLCs³⁹, or the

overall redox state of oocytes as a result of age-related oxidative stress⁴⁴, may comprise SrCl₂-induced oscillation competence.

Our results have important implications for our appreciation of the effect of maternal aging on oocyte health in clinical settings. In cases where egg activation fails following ICSI, many clinics attempt artificial egg activation using Ca²⁺ ionophores such as A23187¹³. However, these treatments only elicit a single rise in Ca²⁺ that does not reflect the series of Ca²⁺ oscillations seen at fertilisation, and may be suboptimal for development¹². Moreover, despite widespread use, the efficacy of these treatments remains unclear. PLC ζ is a physiological agent demonstrated by several laboratories to produce a prolonged series of Ca²⁺ oscillations in mouse and human eggs similar to that of sperm⁵⁴, providing strong indications for its eventual use as a therapeutic to rescue egg activation following failed ICSI in the clinic. By carefully calibrating PLC ζ expression on an egg-by-egg basis using a luciferase tag, we found that young and aged eggs elicit an identical oscillatory signature, as was also the case for ICSI. Thus, at least in mouse, the ability to respond to PLC ζ is not affected by maternal age. These data allude that, should PLC ζ be used in clinical situations, it may not be necessary to titrate according to maternal age, rather that it might be possible to arrive at a universal dose of PLC ζ .

Our data show that egg Ca²⁺ dynamics are largely maintained with advancing maternal age and therefore do not afford a simple explanation for age-related failed activation following assisted reproductive procedures. One possible alternative is that whilst the gross Ca²⁺ oscillatory signature is unchanged, the downstream molecular messengers decoding the Ca²⁺ signal could be compromised. For example, the Ca²⁺ signal is instrumental in inactivation of cytosolic factor (CSF), the cytoplasmic activity that maintains MII arrest. CSF inactivation occurs via Ca²⁺-dependent calmodulin-dependent kinase II activity, and is entirely dependent upon Ca²⁺ oscillations⁵⁵. We consider that, at least in mouse, a defect in Ca²⁺-sensing appears unlikely, since egg activation was unaffected by maternal age. This was even the case for SrCl₂, where oscillation frequency decreased with age, but careful analysis revealed no difference in the temporal dynamics of polar body and pronucleus formation, though we cannot exclude the possibility of more subtle effects of aging on the subsequent kinetics of preimplantation embryonic development. Thus, whilst in our experiments Ca²⁺ dysregulation does not explain failed activation, the question of what causes egg activation failure following ICSI in maternally aged eggs remains unanswered. The role of paternal age in ICSI failure is often hard to analyse in clinical studies^{4–6}. Investigations into PLC ζ protein levels with paternal age would therefore be extremely valuable, as multiple studies show that human sperm with little to no PLC ζ expression fail to initiate egg activation^{56,57}. Alternatively, zinc has recently emerged as an intriguing potential determinant of egg fertilisation success, sperm-egg fusion inducing a series of ‘zinc sparks’ that occur rapidly in response to Ca²⁺ oscillations⁵⁸. Examination of zinc dynamics and other aspects of ionic homeostasis with advancing maternal age may provide insight into why fertilisation failure increases in older women. Finally, though our study shows conclusively that Ca²⁺ dysregulation is minor in a well established model of mammalian oocyte aging, we cannot formally exclude that Ca²⁺ perturbations may be more severe in human eggs from aged patients in suboptimal culture conditions, where fertilisations are performed at various times after egg collection (post ovulatory aging). Future studies of Ca²⁺ responsiveness in failed ICSI cases under controlled conditions in the clinic will thus be invaluable.

In conclusion, our data show advanced maternal age leads to a deterioration in the oocyte’s ability to replenish Ca²⁺ from the extracellular environment, however this has no effect on overall physiological output of Ca²⁺ oscillations. We speculate that, unlike some somatic cells, oocytes may have adapted a defence mechanism to prevent Ca²⁺ dysregulation in the germline, to avoid jeopardising reproductive capacity.

Methods

Egg collection. MII eggs were collected from the oviducts of 3 month old (referred to as ‘young’ eggs) and 12–15 month old (‘naturally-aged’ eggs) female Swiss CD1 mice (Harlan and Charles River Laboratories) following stimulation with pregnant mare’s serum gonadotrophin (i.p., young 5 IU, aged 10 IU) and superovulation with human chorionic gonadotrophin (hCG) (i.p., young 5 IU, aged 10 IU) at 48 hour intervals. Naturally-aged mice were acquired as retired breeders at 7–9 months of age, and housed for a further 5–7 months. Mice were sacrificed 14 hours post-hCG and cumulus masses were released into M2 media containing hyaluronidase (0.3 mg/ml) by rupture of the oviduct with a 27-gauge needle. Cumulus-free eggs were washed through three drops of M2 under mineral oil at 37 °C. All experiments were performed with young and naturally-aged eggs collected contemporaneously. All animal experiments were approved by the Comité Institutionnel de Protection des Animaux du CHUM (CIPA) or the UK Home Office. All animal experiments were performed in accordance with relevant guidelines and regulations of CIPA or the UK Home Office.

Microinjection. Eggs were microinjected using Narishige manipulators mounted on a Leica DMI4000 inverted microscope, as described previously⁵⁹. Briefly, eggs were placed in a drop of M2 under oil and immobilised using a holding pipette. The injection pipette was inserted into the egg cytoplasm and the oolemma breached using a short pulse of negative capacitance from an intracellular electrometer (Warner Instruments). A controlled fixed-pressure injection that displaced a sphere of cytoplasm with a diameter of ~10 μ m (<5% total egg volume) was then delivered using a Picopump (WPI, Sarasota, FL). Following injection, eggs were left to recover for ~30 minutes in M2 under oil at 37 °C.

Intracellular Ca²⁺ measurements. To measure [Ca²⁺]_i, eggs were co-microinjected with Calcium GreenTM-1 dextran (1 mM) and Rhodamine B dextran (1 mM), or incubated in M2 containing Cal-520 AM (5 μ M) for 30 minutes at 37 °C before the experiment. Where necessary, the zona was removed with brief exposure to acidified Tyrode’s solution. Imaging was performed in a glass bottom petri dish heated to 37 °C on a Leica DMI4000 inverted epifluorescence microscope. Images were acquired every 10 seconds and captured for up to

3.5 hours. Ratiometric calculations were performed by dividing Calcium GreenTM-1 dextran values by Rhodamine B after background subtraction. Cal-520 AM fluorescence and the luminescent signal from firefly luciferase were imaged concurrently in the same eggs using a Zeiss Axiovert S100TV microscope. Cal-520 AM was excited from 450–490 nm. The fluorescent and luminescent emission light was collected through the same filter set at 515 nm. The luminescence values presented represent the number of measured photon counts per second.

Ca²⁺ assays and egg activation. To examine [Ca²⁺]_{ER} content, thapsigargin, an inhibitor of the ER Ca²⁺-ATPase, was pipetted directly into the petri dish (final concentration 10 μM). After [Ca²⁺]_i returned to baseline, CaCl₂ (1.7 mM) was added to measure Ca²⁺ influx. ICSI was performed as described⁶⁰ using Piezo-actuated micromanipulation in M2 media containing cytochalasin B (5 μg/ml). Spermatozoa were collected from caudal epididymides of proven-breeder male CD1 mice and a sperm suspension created with 12% PVP₃₆₀ (w/v) (Sigma) to immobilise for Piezo-pulsed decapitation. PLCζ cRNA tagged with firefly luciferase³⁴ was introduced by microinjection and strontium activation achieved by incubating eggs in SrCl₂-containing (10 mM) Ca²⁺-free M2 media.

Data analysis and statistics. Ratiometric images were analysed using Fiji software (Image J) (<http://fiji.sc/Fiji>) and fluorescence intensity from each egg was plotted against time. All changes in [Ca²⁺]_i were statistically analysed using GraphPad Prism Software version 6. The normality of all data sets was assessed using the Shapiro-Wilk test. Data were then analysed using either a Student's two-tailed unpaired t test (parametric data) or a Mann-Whitney test (nonparametric data) as appropriate. Statistical significance was defined as P < 0.05. Actual P values are presented except where P < 0.0001. Data is presented as mean ± standard error (s.e.m.).

References

- Hassold, T. & Hunt, P. To err (meiotically) is human: the genesis of human aneuploidy. *Nat. Rev. Genet.* **2**, 280–291 (2001).
- Kuliev, A., Zlatopolsky, Z., Kirillova, I., Spivakova, J. & Cieslak Janzen, J. Meiosis errors in over 20,000 oocytes studied in the practice of preimplantation aneuploidy testing. *Reprod. Biomed. Online* **22**, 2–8 (2011).
- Howe, K. & FitzHarris, G. Recent insights into spindle function in mammalian oocytes and early embryos. *Biol. Reprod.* **89**, 71 (2013).
- Schwartz, D. & Mayaux, M. J. Female fecundity as a function of age. *N. Engl. J. Med.* **306**, 404–406 (1982).
- Bhattacharya, S., Maheshwari, A. & Mollison, J. Factors associated with failed treatment: an analysis of 121,744 women embarking on their first IVF cycles. *PLoS ONE* **8**, e82249 (2013).
- Devroey, P. *et al.* Female age predicts embryonic implantation after ICSI: a case-controlled study. *Hum. Reprod.* **11**, 1324–1327 (1996).
- Swann, K., Larman, M. G., Saunders, C. M. & Lai, F. A. The cytosolic sperm factor that triggers Ca²⁺ oscillations and egg activation in mammals is a novel phospholipase C: PLCζeta. *Reproduction* **127**, 431–439 (2004).
- Marangos, P., FitzHarris, G. & Carroll, J. Ca²⁺ oscillations at fertilization in mammals are regulated by the formation of pronuclei. *Development* **130**, 1461–1472 (2003).
- Wakai, T. & Fissore, R. A. Ca²⁺ homeostasis and regulation of ER Ca²⁺ in mammalian oocytes/eggs. *Cell Calcium* **53**, 63–67 (2013).
- Kline, D. & Kline, J. T. Repetitive calcium transients and the role of calcium in exocytosis and cell cycle activation in the mouse egg. *Dev. Biol.* **149**, 80–89 (1992).
- Fulton, B. P. & Whittingham, D. G. Activation of mammalian oocytes by intracellular injection of calcium. *Nature* **273**, 149–151 (1978).
- Ozil, J. P., Banrezes, B., Tóth, S., Pan, H. & Schultz, R. M. Ca²⁺ oscillatory pattern in fertilized mouse eggs affects gene expression and development to term. *Dev. Biol.* **300**, 534–544 (2006).
- Ebner, T. *et al.* Live birth after artificial oocyte activation using a ready-to-use ionophore: a prospective multicentre study. *Reprod. Biomed. Online* **30**, 359–365 (2015).
- Thibault, O., Gant, J. C. & Landfield, P. W. Expansion of the calcium hypothesis of brain aging and Alzheimer's disease: minding the store. *Aging Cell* **6**, 307–317 (2007).
- Khachaturian, Z. S. Calcium hypothesis of Alzheimer's disease and brain aging. *Ann. N. Y. Acad. Sci.* **747**, 1–11 (1994).
- Hartmann, H., Eckert, A., Velbinger, K., Rewsin, M. & Müller, W. E. Down-regulation of free intracellular calcium in dissociated brain cells of aged mice and rats. *Life Sci.* **59**, 435–449 (1996).
- Igarashi, H., Takahashi, E., Hiroi, M. & Doi, K. Aging-related changes in calcium oscillations in fertilized mouse oocytes. *Mol. Reprod. Dev.* **48**, 383–390 (1997).
- Takahashi, T., Takahashi, E., Igarashi, H., Tezuka, N. & Kurachi, H. Impact of oxidative stress in aged mouse oocytes on calcium oscillations at fertilization. *Mol. Reprod. Dev.* **66**, 143–152 (2003).
- Jones, K. T. & Whittingham, D. G. A comparison of sperm- and IP₃-induced Ca²⁺ release in activated and aging mouse oocytes. *Dev. Biol.* **178**, 229–237 (1996).
- Merriman, J. A., Jennings, P. C., McLaughlin, E. A. & Jones, K. T. Effect of aging on superovulation efficiency, aneuploidy rates, and sister chromatid cohesion in mice aged up to 15 months. *Biol. Reprod.* **86**, 49 (2012).
- Yun, Y., Lane, S. I. R. & Jones, K. T. Premature dyad separation in meiosis II is the major segregation error with maternal age in mouse oocytes. *Development* **141**, 199–208 (2014).
- Jiao, Z.-X., Xu, M. & Woodruff, T. K. Age-related increase in aneuploidy and alteration of gene expression in mouse first polar bodies. *J. Assist. Reprod. Genet.* **31**, 731–737 (2014).
- Sebestova, J., Danyilevska, A., Novakova, L., Kubelka, M. & Anger, M. Lack of response to unaligned chromosomes in mammalian female gametes. *Cell Cycle* **11**, 3011–3018 (2012).
- American College of Obstetricians and Gynecologists Committee on Gynecologic Practice and Practice Committee. Female age-related fertility decline. Committee Opinion No. 589. *Fertil. Steril.* **101**, 633–4 (2014).
- Figueira, R. de C. S. *et al.* Metaphase II human oocyte morphology: contributing factors and effects on fertilization potential and embryo developmental ability in ICSI cycles. *Fertil. Steril.* **94**, 1115–1117 (2010).
- Jones, K. T., Carroll, J. & Whittingham, D. G. Ionomycin, thapsigargin, ryanodine, and sperm induced Ca²⁺ release increase during meiotic maturation of mouse oocytes. *J. Biol. Chem.* **270**, 6671–6677 (1995).
- Kline, D. & Kline, J. T. Thapsigargin activates a calcium influx pathway in the unfertilized mouse egg and suppresses repetitive calcium transients in the fertilized egg. *J. Biol. Chem.* **267**, 17624–17630 (1992).
- Miao, Y. L., Stein, P., Jefferson, W. N., Padilla-Banks, E. & Williams, C. J. Calcium influx-mediated signaling is required for complete mouse egg activation. *Proc. Natl. Acad. Sci. USA* **109**, 4169–4174 (2012).

29. Cheon, B., Lee, H. C., Wakai, T. & Fissore, R. A. Ca^{2+} influx and the store-operated Ca^{2+} entry pathway undergo regulation during mouse oocyte maturation. *Mol. Biol. Cell* **24**, 1396–1410 (2013).
30. Igusa, Y. & Miyazaki, S. Effects of altered extracellular and intracellular calcium concentration on hyperpolarizing responses of the hamster egg. *J. Physiol.* **340**, 611–632 (1983).
31. Winston, N. J., McGuinness, O., Johnson, M. H. & Maro, B. The exit of mouse oocytes from meiotic M-phase requires an intact spindle during intracellular calcium release. *J. Cell Sci.* **108**(Pt 1), 143–151 (1995).
32. Saunders, C. M. *et al.* PLC zeta: a sperm-specific trigger of Ca^{2+} oscillations in eggs and embryo development. *Development* **129**, 3533–3544 (2002).
33. Rogers, N. T. *et al.* Phospholipase C ζ causes Ca^{2+} oscillations and parthenogenetic activation of human oocytes. *Reproduction* **128**, 697–702 (2004).
34. Swann, K., Campbell, K., Yu, Y., Saunders, C. & Lai, F. A. Use of luciferase chimera to monitor PLC ζ expression in mouse eggs. *Methods Mol. Biol.* **518**, 17–29 (2009).
35. Sfountouris, I. A. *et al.* Artificial oocyte activation to improve reproductive outcomes in women with previous fertilization failure: a systematic review and meta-analysis of RCTs. *Hum. Reprod.* **30**, 1831–1841 (2015).
36. Fernandes, C. B. *et al.* Artificial activation of bovine and equine oocytes with cycloheximide, roscovitine, strontium, or 6-dimethylaminopurine in low or high calcium concentrations. *Zygote* **22**, 387–394 (2014).
37. Tomashov-Matar, R. *et al.* Strontium-induced rat egg activation. *Reproduction* **130**, 467–474 (2005).
38. Méo, S. C., Leal, C. L. V. & Garcia, J. M. Activation and early parthenogenesis of bovine oocytes treated with ethanol and strontium. *Anim. Reprod. Sci.* **81**, 35–46 (2004).
39. Zhang, D. *et al.* Strontium promotes calcium oscillations in mouse meiotic oocytes and early embryos through InsP3 receptors, and requires activation of phospholipase and the synergistic action of InsP3. *Hum. Reprod.* **20**, 3053–3061 (2005).
40. Ducibella, T. *et al.* Egg-to-embryo transition is driven by differential responses to Ca^{2+} oscillation number. *Dev. Biol.* **250**, 280–291 (2002).
41. Shinar, S. *et al.* Total fertilization failure in intra-cytoplasmic sperm injection cycles-classification and management. *Gynecol. Endocrinol.* **30**, 593–596 (2014).
42. Jones, K. T. & Lane, S. I. R. Molecular causes of aneuploidy in mammalian eggs. *Development* **140**, 3719–3730 (2013).
43. Barritt, J. A., Cohen, J. & Brenner, C. A. Mitochondrial DNA point mutation in human oocytes is associated with maternal age. *Reprod. Biomed. Online* **1**, 96–100 (2000).
44. Eichenlaub-Ritter, U., Wieczorek, M., Lücke, S. & Seidel, T. Age related changes in mitochondrial function and new approaches to study redox regulation in mammalian oocytes in response to age or maturation conditions. *Mitochondrion* **11**, 783–796 (2011).
45. Udagawa, O. *et al.* Mitochondrial fission factor Drp1 maintains oocyte quality via dynamic rearrangement of multiple organelles. *Curr. Biol.* **24**, 2451–2458 (2014).
46. Grøndahl, M. L. *et al.* Gene expression profiles of single human mature oocytes in relation to age. *Hum. Reprod.* **25**, 957–968 (2010).
47. Pan, H., Ma, P., Zhu, W. & Schultz, R. M. Age-associated increase in aneuploidy and changes in gene expression in mouse eggs. *Dev. Biol.* **316**, 397–407 (2008).
48. Shen, B. *et al.* Attenuated mesangial cell proliferation related to store-operated Ca^{2+} entry in aged rat: the role of STIM 1 and Orai 1. *Age* **35**, 2193–2202 (2013).
49. Yang, Y. *et al.* Contrasting Patterns of Agonist-induced Store-operated Ca^{2+} Entry and Vasoconstriction in Mesenteric Arteries and Aorta With Aging. *J. Cardiovasc. Pharmacol.* **65**, 571–578 (2015).
50. Tarin, J. J. Potential effects of age-associated oxidative stress on mammalian oocytes/embryos. *Mol. Hum. Reprod.* **2**, 717–724 (1996).
51. Harman, D. Aging: a theory based on free radical and radiation chemistry. *J. Gerontol.* **11**, 298–300 (1956).
52. FitzHarris, G. & Baltz, J. M. Regulation of intracellular pH during oocyte growth and maturation in mammals. *Reproduction* **138**, 619–627 (2009).
53. Carvacho, I., Lee, H. C., Fissore, R. A. & Clapham, D. E. TRPV3 channels mediate strontium-induced mouse-egg activation. *Cell Rep.* **5**, 1375–1386 (2013).
54. Swann, K. & Lai, F. A. PLC ζ and the initiation of Ca^{2+} oscillations in fertilizing mammalian eggs. *Cell Calcium* **53**, 55–62 (2013).
55. Lorca, T. *et al.* Calmodulin-dependent protein kinase II mediates inactivation of MPF and CSF upon fertilization of *Xenopus* eggs. *Nature* **366**, 270–273 (1993).
56. Yoon, S. Y. *et al.* Human sperm devoid of PLC, zeta 1 fail to induce Ca^{2+} release and are unable to initiate the first step of embryo development. *J. Clin. Invest.* **118**, 3671–3681 (2008).
57. Durban, M. *et al.* PLC ζ disruption with complete fertilization failure in normozoospermia. *J. Assist. Reprod. Genet.* **32**, 879–886 (2015).
58. Que, E. L. *et al.* Quantitative mapping of zinc fluxes in the mammalian egg reveals the origin of fertilization-induced zinc sparks. *Nat. Chem.* **7**, 130–139 (2015).
59. FitzHarris, G. A shift from kinesin 5-dependent metaphase spindle function during preimplantation development in mouse. *Development* **136**, 2111–2119 (2009).
60. Yoshida, N. & Perry, A. C. Piezo-actuated mouse intracytoplasmic sperm injection (ICSI). *Nat. Protoc.* **2**, 296–304 (2007).

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Author Contributions

J.H. and G.F. designed the study and wrote the manuscript. J.H. performed experiments shown in Figs 1,2,3,5 and 6. J.H. analysed the results for Figs 1–6. S.N. performed experiments shown in Fig. 3. E.T., D.L., M.N., F.A.L. and K.S. designed and performed the experiments for Fig. 4. All authors reviewed the manuscript.

Additional Information

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