Published in final edited form as:

Mol Reprod Dev. 2011 September ; 78(9): 684–701. doi:10.1002/mrd.21366.

Caffeine alleviates the deterioration of Ca^{2+} release mechanisms and fragmentation of *in vitro* aged mouse eggs

Nan Zhang¹, Takuya Wakai¹, and Rafael. A. Fissore^{1,*}

¹Department of Veterinary and Animal sciences, University of Massachusetts Amherst, Massachusetts 01003, USA

Abstract

The developmental competence of mammalian eggs is compromised by postovulatory aging. We and others found that in these eggs the intracellular calcium ($[\text{Ca}^{2+}]_i$) responses required for egg activation and initiation of development are altered. Nevertheless, the mechanism(s) underlying this defective Ca^{2+} release is not well known. Here, we investigated if the function of IP_3R_1 , the major Ca^{2+} release channel at fertilization, was undermined in *in vitro* aged mouse eggs. We found that in aged eggs IP_3R_1 displayed reduced function, as many of the changes acquired during maturation that enhance IP_3R_1 Ca^{2+} conductivity such as phosphorylation, receptor reorganization and increased Ca^{2+} store content ($[\text{Ca}^{2+}]_{\text{ER}}$) were lost with increasing postovulatory time. IP_3R_1 fragmentation, possibly associated with the activation of caspase-3, was also observed in these eggs. Many of these changes were prevented when the postovulatory aging of eggs was carried out in the presence of caffeine, which minimized the decline in IP_3R_1 function and maintained $[\text{Ca}^{2+}]_{\text{ER}}$ content. Caffeine also maintained mitochondrial membrane potential as measured by JC-1 fluorescence. We therefore conclude that $[\text{Ca}^{2+}]_i$ responses in aged eggs are undermined by reduced IP_3R_1 sensitivity, decreased $[\text{Ca}^{2+}]_{\text{ER}}$ and compromised mitochondrial function, and that addition of caffeine ameliorates most of these aging-associated changes. Understanding the molecular basis of the protective effects of caffeine will be useful in elucidating, and possibly reversing, the signaling pathway(s) compromised by *in vitro* culture of eggs.

Keywords

IP_3R_1 ; postovulatory egg aging; anti-aging treatment; caffeine; calcium homeostasis; fertilization; oocyte quality

INTRODUCTION

Ovulated mammalian eggs are arrested at the metaphase stage of the second meiotic division (MII), stage at which they will remain until fertilization. Successful embryo development is mostly attained when fertilization takes place within 10 hrs of ovulation (Fissore et al. 2002; Marston and Chang 1964; Sakai and Endo 1988; Tarin et al. 1999; Yanagimachi and Chang 1961), as after this period a time-dependent aging process sets in that compromises development; these changes occur more rapidly if eggs remain in the oviduct (Smith and Lodge 1987; Wilcox et al. 1998; Xu et al. 1997; Zackowski and Leon 1988) but they also occur when eggs are cultured *in vitro* (Trownson et al. 1982; Abbott et al. 1998). Postovulatory aging affects many aspects of egg function, including the ability to be fertilized (Badenas et al. 1989), to maintain the MII arrest (Kikuchi et al. 1995), and to

*Address for Correspondence: Department of Veterinary and Animal Sciences, ISB-661 North Pleasant Street, University of Massachusetts Amherst, Massachusetts 01003, USA. Tel: (413) 545-5548; Fax: (413) 545-6326; rfissore@vasci.umass.edu.

undergo development upon fertilization (Tarin et al. 1999; Gordo et al. 2002). Together, these defects undermine reproductive efficiency and compromise the health of the offspring (Tarin et al. 1999).

The prospect of postovulatory aging of eggs under *in vitro* conditions has increased significantly with the advent of assisted reproductive technologies (ART). While the application ART has revolutionized the treatment of infertility, these techniques require gametes being maintained under *in vitro* conditions for prolonged periods of time, which might later compromise embryo development. For example, research shows that prolonged culture of matured oocytes *in vitro* prior to *in vitro* fertilization (IVF) or intracytoplasmic sperm injection (ICSI) gradually affects the quality of both human and mouse eggs and impairs subsequent embryonic development (Fukuda et al. 1992; Yanagida et al. 1998). It is therefore important to elucidate the cellular functions and underlying mechanisms that are affected in eggs during *in vivo* and *in vitro* aging so that corrective approaches can be developed.

Egg activation, the first stage of embryo development, is triggered in all species studies to date by an increase in the intracellular concentration of free calcium ($[Ca^{2+}]_i$). In mammals, the Ca^{2+} signal adopts a pattern of repeated $[Ca^{2+}]_i$ rises that is also referred to as $[Ca^{2+}]_i$ oscillations (Ducibella and Fissore 2008; Miyazaki 2006; Stricker 1999). These oscillations appear to be initiated by the release of a sperm-specific phospholipase C (PLC) isoform, PLC ζ (Saunders et al. 2002), which is thought to hydrolyze phosphatidylinositol 4,5-bisphosphate (PIP₂) and produce inositol 1,4,5-trisphosphate (IP₃), the ligand of IP₃Rs, the Ca^{2+} channels that are predominantly located on the endoplasmic reticulum (ER), the eggs' main Ca^{2+} reservoir. During maturation, IP₃R1, the most abundant isoform in oocytes, undergoes modifications that optimize Ca^{2+} release at the time of fertilization. Some of these changes include an increase in the total amount of IP₃R1 (Parrington et al. 1998; Xu et al. 2003), cortical redistribution of the receptor, formation of IP₃R1 clusters (Fissore et al. 1999; Mehlmann et al. 1996), and enhanced receptor phosphorylation (Jellerette et al. 2004; Lee et al. 2006). The concentration of Ca^{2+} of the ER ($[Ca^{2+}]_{ER}$) also increases during maturation (Jones et al. 1995; Kline 2000) and may further potentiate Ca^{2+} release in MII eggs. It is therefore possible that the changes acquired during oocyte maturation that enhance IP₃R1-mediated Ca^{2+} release are reversed during postovulatory aging, thereby compromising Ca^{2+} release and egg activation. It is known that a defective Ca^{2+} signal compromises embryonic developmental competence, as demonstrated by the elegant studies of Ozil and coworkers (Ducibella et al. 2006; Ozil et al. 2006; Ozil et al. 2005). In those studies, administration of fewer $[Ca^{2+}]_i$ pulses produced fewer pronucleus (PN) zygotes and greatly reduced development to the blastocysts stage.

Changes in other biochemical functions may also underlie the loss of fertilizability and developmental competence of aged mammalian eggs. For instance, studies show that aged eggs display reduced levels of P34^{cdc2}/cyclin B (MPF) and MAP kinase activity (Kikuchi et al. 1999), increased rates of chromatid disjunction (Eichenlaub-Ritter et al. 1988; Mailhes et al. 1998; Rodman 1971; Steuerwald et al. 2005), loss of cortical spindle localization (Szollosi 1971; Van Wissen et al. 1991), decreased mitochondrial function (Wilding et al. 2001) and abnormal actin distribution (Kim et al. 1996). Given the importance of these cellular mechanisms for normal embryo development, attempts have been made to delay or reverse these changes. For example, it is known that oxidative stress is detrimental for mitochondrial function (reviewed in Roede and Jones 2010) and the use of Dithiothreitol (DTT), which lessens the effects of oxidation, reportedly enhances fertilization of aged oocytes and blastocyst development of aged zygotes (Rausell et al. 2007; Tarin et al. 1998). Nitric oxide was also shown to delay several aging-related phenomena such as hardening of the zona pellucida and spontaneous cortical granule exocytosis, although the mechanism(s)

of action of this compound remains unclear (Goud et al. 2005). Caffeine, a well-known phosphodiesterase inhibitor (Essayan 2001), has recently been used to prevent the loss of developmental competence of *in vitro* aged eggs. Reports showed that the presence of caffeine during culture led to higher rates of blastocyst formation (Maalouf et al. 2009) and increased cell numbers in blastocysts (Lee and Campbell 2008) of ovine eggs, although another study failed to notice beneficial effects of caffeine supplementation on developmental ability in aged porcine oocytes (Iwamoto et al. 2005). The specific molecular mechanism(s) modified by caffeine in aged eggs remains to be determined.

This study focuses on understanding the mechanisms of Ca^{2+} release and homeostasis that are affected during postovulatory aging. We also examined the effect of caffeine on these parameters. Our results show that postovulatory egg aging negatively affects $\text{IP}_3\text{R1}$ function, essentially erasing the optimization attained during maturation. We found that addition of caffeine delayed/reversed many of these detrimental changes, thereby restoring $[\text{Ca}^{2+}]_i$ responsiveness in aged eggs.

RESULTS

$\text{IP}_3\text{R1}$ -mediated Ca^{2+} release is compromised in aged eggs

Fertilized aged eggs show reduced developmental capacity (Tarin et al. 1999; Tarin et al. 2000; Wilcox et al. 1998) and altered Ca^{2+} homeostasis (Gordo et al. 2002; Igarashi et al. 1997; Jones and Whittingham 1996; Takahashi et al. 2009; Takahashi et al. 2000). Regarding the latter, postovulatory aged eggs exhibit reduced $\text{IP}_3\text{R1}$ mediated Ca^{2+} release, although it is unclear whether this is due to direct changes on $\text{IP}_3\text{R1}$ or caused by factors that indirectly affect the function of the receptor. To extend those studies, we first examined whether $\text{IP}_3\text{R1}$ mediated Ca^{2+} release was disturbed in *in vitro* aged mouse eggs, our model of choice, as most previous studies have evaluated responses in *in vivo* aged mouse eggs (Igarashi et al. 1997; Jones and Whittingham 1996). To accomplish this, cytosolic IP_3 levels were increased in recently ovulated MII eggs (14hrs post hCG) and in *in vitro* aged eggs (38hrs post hCG). UV light pulses of different duration were used to release IP_3 from c IP_3 . Our results (Fig. 1A,B) show that the two shorter UV pulses, 0.01 and 0.05s, resulted in severely impaired $[\text{Ca}^{2+}]_i$ responses in aged eggs ($P < 0.01$), whereas the highest IP_3 release evoked by the 0.25s UV pulse induced $[\text{Ca}^{2+}]_i$ responses of comparable amplitude ($P > 0.05$) but dissimilar duration ($P < 0.01$) in fresh MII and aged eggs, respectively. Thus, our results suggest that *in vitro* postovulatory aging of eggs impairs $\text{IP}_3\text{R1}$ mediated Ca^{2+} release.

$[\text{Ca}^{2+}]_{\text{ER}}$ and $\text{IP}_3\text{R1}$ sensitivity are decreased in aged eggs

A previous study observed reduced $\text{IP}_3\text{R1}$ -mediated Ca^{2+} release in aged eggs, although it was attributed to lower $[\text{Ca}^{2+}]_{\text{ER}}$ rather than to changes in $\text{IP}_3\text{R1}$ sensitivity/function (Takahashi et al. 2000). We therefore estimated $[\text{Ca}^{2+}]_{\text{ER}}$ using TG, a sarco/endoplasmic reticulum Ca^{2+} ATPase inhibitor, which causes emptying of the Ca^{2+} stores (Thastrup et al. 1990). We found that while aged eggs showed lower $[\text{Ca}^{2+}]_{\text{ER}}$ than recently ovulated eggs (Fig. 2A, B; $P < 0.05$), the magnitude of the difference, ~35%, seemed unlikely to account for the disparity observed in the $[\text{Ca}^{2+}]_i$ responses induced by threshold IP_3 concentrations, which were ~70% larger in fresh than in aged eggs.

While the above results did not exclude the possibility that $[\text{Ca}^{2+}]_{\text{ER}}$ might affect $[\text{Ca}^{2+}]_i$ responses in aged eggs, they raised the prospect that other factors may also be at play. We first examined whether the sensitivity of $\text{IP}_3\text{R1}$ changed in *in vitro* aged eggs. To test this, we released IP_3 at threshold concentrations, which were obtained using 0.05s UV pulses, and at near saturating concentrations of IP_3 , which were attained by 0.25s UV pulses. We then calculated what percentage of the Ca^{2+} released by the saturating IP_3 pulse was

released by the threshold pulse of IP₃. While 14/15 fresh MII eggs displayed a [Ca²⁺]_i rise after the low cIP₃ pulse, only 8/15 aged eggs did so; the larger IP₃ pulse induced a [Ca²⁺]_i rise in all eggs (representative traces in Fig. 3A, B). Plotting the mean amplitudes of the [Ca²⁺]_i responses showed that the short UV pulse produced a nearly saturating [Ca²⁺]_i response in fresh eggs, whereas in aged eggs it caused only ~40% of the amplitude of the [Ca²⁺]_i response induced by the saturating IP₃ pulse (Fig. 3C, D, E; P<0.01). Furthermore, calculation of the EC₅₀, i.e. the duration of the UV pulse required to attain half-maximum Ca²⁺ release, showed that this value was of 0.023s for young eggs vs. 0.080s for aged eggs (Fig. 3D; P<0.05). Collectively, our data suggest that IP₃R1 sensitivity is reduced in aged eggs.

Postovulatory egg aging induces IP₃R1 biochemical modifications

We next examined whether the biochemical changes that IP₃R1 undergoes during maturation are reversed during postovulatory aging. We first examined whether the concentration of IP₃R1 changes during *in vitro* culture. We pursued this possibility since IP₃R1 contains a caspase-3 cleavage site in its C-terminal end (Hirota et al. 1999), and caspase activity is present in aged mouse eggs (Gordo et al. 2002; Papandile et al. 2004; Perez et al. 1999). Moreover, in a previous study we found that along with wild type IP₃R1, aged mouse eggs display a ~90 kDa band, which is assumed to correspond to the C-terminal end of IP₃R1 (Verbert et al. 2008). Here, we show that both non-fragmented and fragmented aged eggs display the IP₃R1 cleavage product, although it is more abundant in fragmented eggs (Fig. 4A; P<0.05 and P<0.01, respectively). Remarkably, despite the presence of the cleaved product, the total concentration of wild type IP₃R1 was not affected in aged eggs (Fig. 4A). We also investigated whether IP₃R1 phosphorylation, which occurs during maturation (Lee et al. 2006), was abrogated during postovulatory aging. The MPM-2 antibody was used to detect IP₃R1 phosphorylation, as it recognizes phosphorylations associated with M-Phase kinases (Jellerette et al. 2004; Lee et al. 2006). While fresh MII eggs exhibited MPM-2 reactivity corresponding to IP₃R1, this immunoreactivity was severely reduced in both groups of aged eggs (Fig. 4B; P<0.05, P<0.01, respectively). Lastly, we examined the distribution of IP₃R1 using immunofluorescence (IF). As expected, in fresh MII eggs, IP₃R1 was present throughout the ooplasm corresponding with the distribution of the ER including the formation of conspicuous cortical clusters (Fig. 4C; left panels). Conversely, aged eggs lacked these clusters, and the receptor showed a more diffuse distribution (Fig. 4; right panels). Collectively, the data show that several biochemical and cellular properties of IP₃R1 are negatively regulated during postovulatory egg aging.

Caffeine prevents the decrease in IP₃ mediated Ca²⁺ release and prevents the loss of IP₃R1 sensitivity associated with egg aging

Recent studies have reported that supplementation of the culture media with caffeine reduces changes associated with postovulatory egg aging (reviewed in Miao et al. 2009). Results from those studies using mammalian eggs showed that caffeine is effective at slowing down the deterioration of cellular functions, including maintaining normal levels of MPF activity (Kikuchi et al. 2000; Kikuchi et al. 1999), improving nuclear remodeling after somatic nuclear transfer (Iwamoto et al. 2005) and maintaining acceptable rates of fertilization after IVF (Manonmani et al. 2004). It is therefore of interest to examine whether caffeine supplementation could maintain IP₃R1 function in aged eggs. IP₃R1 function was probed with cIP₃ as previously described, and eggs were aged in the presence or absence of 5 mM caffeine as well as in the presence of colcemid, a microtubule polymerization inhibitor that preserves [Ca²⁺]_i oscillatory activity (Jellerette et al. 2004) and inhibits fragmentation following activation of aged eggs (Fissore et al. 2002). Our results show that caffeine-treated aged eggs displayed [Ca²⁺]_i responses that were comparable to those

initiated in fresh eggs, whereas untreated or colcemid-treated eggs showed reduced Ca^{2+} parameters (Fig. 5A).

We next examined whether caffeine directly modulated $\text{IP}_3\text{R1}$ sensitivity in aged eggs. To test this, we used fresh and aged eggs as well as eggs aged in the presence of caffeine. IP_3 release was accomplished from cIP_3 as previously described. The near threshold pulse of IP_3 induced the expected large $[\text{Ca}^{2+}]_i$ rise in fresh eggs, although it caused minimal release in aged eggs; caffeine treated eggs exhibited responses that were indistinguishable from those of fresh eggs (Fig. 5B). The $[\text{Ca}^{2+}]_i$ responses induced by near saturating IP_3 concentrations were similar in all groups examined (Fig. 5C), consistent with previous results. Estimation of $\text{IP}_3\text{R1}$ sensitivity in these eggs, which was performed according to the relative amplitude of the $[\text{Ca}^{2+}]_i$ responses induced by two dissimilar IP_3 concentrations, showed that caffeine supplementation indeed avoided the decline of this parameter in *in vitro* cultured eggs (Fig. 5D, $P < 0.01$). Altogether, our results show that caffeine prevents some of the detrimental effects of aging on the $\text{IP}_3\text{R1}$ -mediated Ca^{2+} signal.

Exposure to caffeine maintains $[\text{Ca}^{2+}]_i$ oscillatory responses in aged eggs and prevents the decrease in $[\text{Ca}^{2+}]_{\text{ER}}$

To test whether caffeine could maintain the ability of eggs to mount $[\text{Ca}^{2+}]_i$ oscillations after *in vitro* culture, we examined the $[\text{Ca}^{2+}]_i$ responses initiated by injection of $0.5 \mu\text{g}/\mu\text{l}$ PLC ζ mRNA in the presence and absence of this compound. In fresh eggs, PLC ζ induced $[\text{Ca}^{2+}]_i$ responses started within 20 min post injection, while in untreated aged eggs and in colcemid-treated eggs $[\text{Ca}^{2+}]_i$ responses started nearly 80 min after injection (Fig. 6A, B; $P < 0.01$). PLC ζ -induced $[\text{Ca}^{2+}]_i$ responses in caffeine-treated eggs approximated those of fresh eggs in terms of the duration of the 1st rise (Fig. 6B; $P > 0.05$), and time to first rise, which while it was not as early as those in fresh eggs, occurred significantly earlier than in aged- or colcemid-treated eggs ($P < 0.05$, data not shown). Furthermore, caffeine supplementation maintained near normal levels of $[\text{Ca}^{2+}]_{\text{ER}}$ in *in vitro* cultured eggs (Fig. 6C, D; $P > 0.05$). Together, these results show that caffeine positively influences several aspects of Ca^{2+} homeostasis in *in vitro* aged eggs.

Caffeine modifies $\text{IP}_3\text{R1}$ distribution and maintains mitochondrial activity in aged eggs

Given that *in vitro* cultured eggs show loss of $\text{IP}_3\text{R1}$ MPM-2 reactivity and abnormal cellular distribution, we tested the possibility that caffeine might also alleviate some of these changes. Caffeine did not prevent the loss of $\text{IP}_3\text{R1}$ MPM-2 reactivity associated with eggs aging (data not shown), which is consistent with our observation that it did not prevent the gradual decline of MPF and MAP kinase activities (data not shown). Caffeine also failed to restore the ER-like network distribution and cortical cluster organization characteristic of $\text{IP}_3\text{R1}$ in fresh eggs, although it induced $\text{IP}_3\text{R1}$ accumulation in large patches, ~ 5 to $10 \mu\text{m}$ in diameter, some of which were localized to the egg's cortex (Fig. 7A).

Actin plays an essential role in cortical granule distribution of mouse eggs (Abbott et al. 2001; Connors et al. 1998; Wessel et al. 2002) and on the ER cortical clusters organization in MII eggs (FitzHarris et al. 2007; Mehlmann et al. 1996). Research has shown that the thick actin cap that normally lies on top of the spindle in MII eggs is lost in aged eggs (Kim et al. 1996; Webb et al. 1986), suggesting actin dysfunction, and we examined whether caffeine could prevent these changes. Actin distribution was followed with Alexa-phalloidin, which was injected into eggs immediately before observation. As shown in Figure 7B, a dense actin cap overlaid the MII spindle area, and a continuous actin ring delineated the circumference of fresh eggs. Neither of these features was present in aged eggs, and while the addition of caffeine managed to maintain the polarized distribution of actin, it did not conserve the fine cortical actin ring. Altogether, the results show that post

ovulatory aging alters IP₃R1 distribution and actin organization, and that these changes are not prevented by addition of caffeine.

Mitochondrial activity is essential for cell function and oocytes are not an exception to this rule (Dumollard et al. 2004). Moreover, impaired mitochondrial function and decreased intracytoplasmic levels of ATP have been reported in *in vitro* aged human and mouse eggs (Chi et al. 1988; Wilding et al. 2001). We therefore investigated whether the addition of caffeine maintained mitochondrial membrane potential of ($\Delta\phi_m$), which we used as an indication of this organelle's activity. Membrane potential was measured using JC-1 (5, 5', 6, 6'-tetrachloro-1, 1', 3, 3'-tetraethylbenzimidazolylcarbocyanine iodide), a lipophilic cationic dye that enters and accumulates in the mitochondrial matrix and shifts emission spectra as $\Delta\phi_m$ increases (Reers et al. 1995; Reers et al. 1991). Following incubation with JC-1, images of fresh, aging and caffeine-treated aging eggs were acquired using the same confocal parameters. In fresh MII eggs, mitochondria with high $\Delta\phi_m$, which form red fluorescence aggregates, distributed to the subcortical region in the shape of a ring. In aged eggs, this distribution was lost and it became more diffuse (Fig. 7C). In ~35%±5 of the caffeine-treated eggs, the mitochondria with high $\Delta\phi_m$ maintained the subcortical ring distribution. In addition, the size of individual aggregates and the red to green fluorescence intensity ratio, which have been used as indicators of mitochondrial activity (Van Blerkom et al. 2002; Wilding et al. 2001), were comparable to those of fresh MII eggs (Fig. 7D, E $p<0.05$). To confirm that JC-1 fluorescence ratio was an approximate reflection of $\Delta\phi_m$, FCCP (Carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone), a well-known mitochondrial uncoupler, was used to dissipate the $\Delta\phi_m$. Addition of FCCP caused rapid decrease (within 10 min) in the fluorescence ratio of all eggs (Fig. 7A, right panel) demonstrating that JC-1 reports mitochondrial membrane potential in mouse eggs. Collectively, these results show that addition of caffeine can prevent the deterioration of mitochondrial function of postovulatory aging eggs.

Effects of caffeine treatment on activation and development on aged mice eggs

To investigate how caffeine-treated mouse eggs would respond to activation and initiation of development, we induced parthenogenetic activation. Egg activation was accomplished by exposing eggs to Ionomycin (Iono) for 5 min in Ca²⁺-free media followed by 4 hrs treatment with cycloheximide (CHX), after which we monitored for signs of activation. By 4 hrs post activation, the majority of fresh MII eggs had extruded the 2nd polar body and displayed pronuclear (PN) formation; most of these eggs later progressed to the 2-cell stage (Table 1). Conversely, by the same time, untreated aged eggs either did not exit MII or fragmented, and the majority of these eggs were fragmented by 24 hrs. The addition of caffeine avoided fragmentation and most eggs activated, although instead of releasing a polar body, they cleaved immediately to the 2-cell stage; these embryos remained at the 2-cell stage when visualized 24 hrs later (Fig. 8A).

In an attempt to prevent the immediate cleavage and force PN formation, activated caffeine-treated eggs were treated with cytochalasin B (Cyto B), a microfilament inhibitor that blocks cytokinesis. As expected, while the treatment prevented polar body extrusion in fresh eggs and delayed 2-cell cleavage in caffeine-treated eggs, it failed to allow PN formation in this group, which cleaved immediately after removal of cyto B (Table 2). Nevertheless, caffeine prevented embryo fragmentation and ~12% of the embryos cleaved progressed to the 4-cell stage ($P>0.05$, data not shown); the rest of the embryos arrested at the 2-cell stage or underwent fragmentation by 48 hrs.

DISCUSSION

The mechanisms that may underlie the defective $[Ca^{2+}]_i$ responses in postovulatory *in vitro* aged mouse eggs were examined in the present study. Our results show that: 1) IP₃R1 mediated Ca^{2+} release is impaired in aged eggs; 2) $[Ca^{2+}]_{ER}$ content is reduced in these cells; 3) changes in IP₃R1 organization and biochemical properties acquired during maturation that enhance IP₃R1 function are lost during prolonged *in vitro* culture; 4) caffeine supplementation delays some of the detrimental changes in IP₃R1 and mitochondrial functions associated with *in vitro* culture. Collectively, the data show that post ovulatory aging of mouse eggs causes alteration of Ca^{2+} homeostasis, IP₃R1 dysfunction and mitochondrial failure, factors all likely to compromise the developmental competence of these eggs.

Altered Ca^{2+} homeostasis and $[Ca^{2+}]_i$ responses associated with egg aging have been documented in several reports, although from those studies it was difficult to discern the mechanism(s) responsible for the changes. One study using *in vivo* aged eggs noted that sperm initiated $[Ca^{2+}]_i$ oscillations were of lower amplitude and higher in frequency than those induced in recently ovulated MII eggs (Igarashi, et al., 1997). It was found that $[Ca^{2+}]_{ER}$ content was reduced in aged eggs and this was attributed to decreased SERCA activity caused by limited ATP supplies (Igarashi et al. 1997; Takahashi et al. 2009). In previous reports we observed that $[Ca^{2+}]_i$ oscillations initiated by injection of agonists ceased prematurely *in vitro* aged eggs, which also suggests reduced $[Ca^{2+}]_{ER}$ content (Gordo et al. 2002; Verbert et al. 2008). While we did not examine the cause of reduced $[Ca^{2+}]_{ER}$ levels, we detected increased presence of a C-terminal fragment of IP₃R1 (Verbert, et al., 2008), which is consistent with the reported increase in caspase-3 activity in aged and fragmented mouse eggs (Gordo et al. 2002; Papandile et al. 2004; Perez et al. 1999). In somatic cells, the presence of truncated IP₃R1s results in a “leaky channel” that increases Ca^{2+} flux into the cytoplasm causing depletion of $[Ca^{2+}]_{ER}$, and possibly underlying other changes associated with cell death (Joseph and Hajnoczky 2007; Khan et al. 2007; Kothakota et al. 1997). In mouse eggs, consistent with these results, over-expression of an mRNA encoding for the C-terminal end of IP₃R1 caused reduction of the $[Ca^{2+}]_{ER}$ content and increased the rate of egg fragmentation (Verbert et al. 2008). The levels of IP₃R1 C-terminal fragment found in our study were low compared to the total amount of intact IP₃R1, and thus it is unclear whether this mechanism plays a role in disturbing Ca^{2+} homeostasis in aged eggs.

Our studies also found abnormal IP₃R1 function in aged mouse eggs. Using a wide range of cIP₃ concentrations in *in vitro* aged eggs vs. fresh MII eggs, we found a significant reduction in the proportion of Ca^{2+} released by threshold concentrations of cIP₃ in aged eggs. Several factors may be responsible for the decreased IP₃R1 sensitivity, and an obvious one is the reduced $[Ca^{2+}]_{ER}$ content that we observed in aged eggs. $[Ca^{2+}]_{ER}$ content reportedly affects IP₃R1 function in various cell types (Horne and Meyer 1995; Missiaen et al. 1991; Oldershaw and Taylor 1993; Sienaert et al. 1997), and it was therefore surprising that despite reporting reduced $[Ca^{2+}]_{ER}$ content in aged eggs, a previous study failed to detect defects in IP₃R1 sensitivity (Takahashi, et al., 2000). It is worth noting however that other factors that we observed in our study such as decreased IP₃R1 MPM-2 reactivity and altered sub-cellular distribution of the receptor may compromise IP₃R1 function in aged eggs. Altogether these findings indicate that the sensitivity of IP₃R1 is compromised in *in vitro* aged eggs, and that several cellular and biochemical factors might contribute to undermine its function.

Caffeine supplementation during the aging period was able to prevent many of the changes associated with loss of IP₃R1 function. For instance, caffeine supplementation avoided loss

of IP₃R1 sensitivity, depletion of [Ca²⁺]_{ER} and modified the altered actin organization. Interestingly, caffeine failed to prevent the loss of MPM-2 IP₃R1 phosphorylation and IP₃R1 organization, although it caused the formation of large IP₃R1 clusters and patches of throughout the ooplasm, including the cortex. It is unknown whether the reorganization of IP₃R1 caused by caffeine contributed to prevent the loss of IP₃R1 sensitivity in aged eggs, although MPM-2 receptor phosphorylation does not seem to underlie the positive effects of caffeine on IP₃R1 function.

The precise molecular pathway(s) by which caffeine ameliorates the effects of aging in eggs is not known. In previous studies, caffeine was shown to prevent the decline in the activity of MPF and MAPK that is associated with egg aging during *in vitro* culture (Kikuchi et al. 2002; Kikuchi et al. 2000) and support high rates of *in vitro* development to the blastocysts stage of nuclear transfer embryos (Kwon et al. 2008; Maalouf et al. 2009). In the present study we did not observe these effects of caffeine on kinase activity, and given that aged eggs treated with colcemid, which maintains high MPF activity (Sablina et al. 2001), failed to show improved [Ca²⁺]_i responses, we conclude that this is not the mechanism of action of caffeine.

While caffeine did not maintain M-kinase activities, it did preserve mitochondrial function, as assessed by JC-1 ratio of red/green fluorescence and size of red fluorescence mitochondrial clusters. Both values were higher in caffeine-treated aged eggs than in aged untreated eggs. These data are consistent with previous findings suggesting that impaired mitochondrial function is one of the molecular mechanisms underlying the developmental incompetence of aged mouse and human eggs (Chi et al. 1988; Wilding et al. 2001). It is therefore possible that under caffeine treatment mitochondrial function in aged eggs is maintained thereby providing adenosine triphosphate (ATP) for [Ca²⁺] homeostasis during fertilization and development (Dumollard et al. 2004; Liu et al. 2001); our observation that [Ca²⁺]_{ER} levels are maintained in caffeine-treated aged eggs supports this contention.

It is well established that *in vitro* culture is associated with increased levels of reactive oxygen species (ROS) that compromise mitochondrial function (Thouas et al. 2004; Wang et al. 2009). Consistent with this possibility, exposure to various kinds of ROS, including superoxide, hydrogen peroxide, and hypochlorous acid accelerates the aging phenomena in eggs (Goud et al. 2008). Furthermore, aged eggs exhibit reduced levels of glutathione (GSH), a reducing compound that prevents the increase in the levels of ROS (Boerjan and de Boer 1990; Igarashi et al. 2005; Tarin 1996), and incubation of eggs with reducing agents such as DTT reportedly alleviated the effects of aging on fertilization rates and embryo fragmentation (Rausell et al. 2007; Tarin et al. 1998). While it is unknown if caffeine specifically prevents oxidative stress in eggs, it is worth noting that caffeine has been shown to have antioxidant properties (Azam et al. 2003; Mukhopadhyay et al. 2003) and prevented increases in ROS levels following X or γ-radiation in somatic cells and bacteria (Kamat et al. 2000; Kesavan and Powers 1985). It is therefore possible that caffeine protects the mitochondria from the detrimental effects of ROS accumulating in aged eggs.

Besides the aforementioned biochemical improvements, we observed that caffeine-treated aged eggs showed enhanced egg activation rates and decreased fragmentation following application of the activation stimulus. While caffeine prevented fragmentation, it failed to support the normal phenotype of activation, as caffeine-treated aged eggs underwent immediate cleavage to the 2-cell stage, a result that was not altered even by the addition of CytoB for 4 hrs. Rapid cleavage to the 2-cell stage has been observed following activation of aged mouse eggs (Webb et al. 1986) and *in vitro* aged porcine oocytes (Kim et al. 1996), which is consistent with the loss of cortical location of the spindle and altered cytoskeletal organization observed in aging eggs (Szollosi 1971; Van Wissen et al. 1991; Webb et al.

1986). Our results showing that caffeine was unable to maintain actin distribution and the peripheral location of spindles and chromosomes in aging eggs are consistent with these activation results. Remarkably, despite the early cleavage, caffeine-treated embryos failed to actively divide and in most cases did not progress beyond the 4-cell stage, although they remain intact and did not fragment for at least 48 hrs. While the haploid status of these embryos is known to compromise pre-implantation development (Kaufman and Sachs 1976), the failure of these embryos to progress beyond the 4-cell stage was unexpected. It is noteworthy, that while caffeine did not support complete pre-implantation development, control aged eggs in the absence of caffeine failed to show any signs of activation and within a few hrs underwent fragmentation. These results suggest that while aging in the presence of caffeine is capable of preventing widespread deterioration of eggs and zygotes, there are several other mechanisms that are required for embryo development such as cytoskeletal organization, MPF activity and others functions that we are still ignorant of, which are not protected by caffeine addition. In this regard, it is worth noting that a recent manuscript reported the birth of pups from ICR and B6D2F1 aged eggs cultured in 10 mM caffeine (Ono et al. 2011). In that study, caffeine extended full developmental potential by 2 (ICR) to 4 hrs (B6D2F1), or to 28 and 34 hrs post hCG, respectively. In our study, the effects of caffeine were evaluated in eggs aged to 36 hrs post hCG of the CD-1 strain, which is equivalent to ICR. As shown here, by 36 hrs post hCG, caffeine was unable to rescue pre-implantation embryo development.

In summary, here we show that IP₃R1 and mitochondrial dysfunction may underlie, at least in part, the defective Ca²⁺ release and developmental competence of post-ovulatory *in vitro* aged mouse eggs. We identified several mechanisms that affect IP₃R1s sensitivity and show that IP₃R1 inactivation can be prevented by caffeine supplementation. Identification of the molecular mechanism(s) by which caffeine prevents the detrimental effects of aging as well as the elucidation of the pathways that are not modified by it will provide deeper understanding of the mechanisms underlying the *in vitro* aging of eggs. It is possible that this knowledge may be applicable to oocytes that age naturally in the follicles of older females, which will serve to prevent the early demise of fertility in some individuals.

MATERIAL AND METHODS

Animal care and welfare

Animals used for gamete collections herein were handled following the National Research Council's Animal Care and Welfare Guidelines. These procedures were approved by the Institutional Animal Care and Use Committee at the University of Massachusetts.

Egg collection and culture conditions

Superovulation was carried out as previously described (Gordo et al. 2002). CD-1 female mice, 6- to 8- week-old, were injected with 5 IU of pregnant mare serum gonadotropin (PMSG; Sigma, St Louis, MO; all chemicals were purchased from Sigma unless otherwise specified). MII eggs were obtained from oviducts 12–14 hrs after injection of 5 IU hCG, which was administered 46–48 hrs after PMSG injection. Eggs were released into HEPES-buffered tyrode-lactate solution (TL-HEPES) supplemented with 5% heat-treated fetal calf serum (FCS; Gibco BRL, Grand Island, NY) followed by treatment with 0.1% bovine testes hyaluronidase for 3–5 min to remove cumulus cells. MII eggs were thoroughly washed and transferred into 50 µl drops of KSOM (Potassium Simplex Optimized Medium; Specialty Media, Phillipsburg, NJ) containing 0.1% polyvinyl alcohol (PVA) under paraffin oil at 36.5°C and in a humidified atmosphere containing 5% CO₂. *In vitro* aging was accomplished by extending the culture time of eggs after collection in KSOM medium to 24 hrs.

Microinjection and preparation of PLC ζ cRNA

Microinjection was performed as previously described (Gordo et al. 2002). In brief, eggs were microinjected under a Nikon Diaphot microscope (Nikon, Inc., Garden City, NY) using Narishige manipulators (Medical Systems Corp., Great Neck, NY). Reagents were loaded into glass micropipettes by aspiration and delivered by pneumatic pressure (PLI-100 picoinjector, Harvard Apparatus, Cambridge, MA). The injection volume was ~7–12 pl (1–3% of the total volume of the egg). For mRNA preparation, the full-length sequence of mouse PLC ζ (a kind gift from Dr K. Fukami, Tokyo University of Pharmacy and Life Science, Japan) within a pBluescript plasmid was *in vitro* transcribed using the T7 mMESSAGE mMACHINE Kit followed by poly A tailing (Ambion, Austin, TX), as reported previously (Kurokawa et al. 2004). Concentrated mRNA (2 μ g/ μ l) was heated for 3 min at 85 °C and diluted to 0.05 μ g/ μ l in RNAase free water before microinjection.

[Ca²⁺]_i imaging

[Ca²⁺]_i measurements were carried out as previously described, maximum of 20 eggs could be monitored together each time (Kurokawa and Fissore 2003). Eggs were loaded with 1.25 μ M fura-2 AM (Molecular Probes, Eugene, OR) supplemented with 0.02% pluronic acid (Molecular Probes, Eugene, OR) for 20 minutes (min) at room temperature (RT). Eggs were then thoroughly washed and attached to glass-bottom dishes (MatTek Corp, Ashland, MA) in drops of FCS-free TL-HEPES under mineral oil, because eggs incline to stick to glass or plastic surface in the absence of protein source. [Ca²⁺]_i values were monitored using a Nikon Diaphot microscope fitted for fluorescence measurements. Eggs were simultaneously monitored using the software SimplePCI (C-Imaging System, Cranberry Township, PA), which controls a filter wheel rotating between excitation wavelengths of 340 and 380 nm illuminated by a 75 W Xenon arc lamp. Emitted light above 510 nm was collected by a cooled Photometrics SenSys CCD camera (Roper Scientific, Tucson, AZ) every 20 seconds (s) and used to calculate fluorescence ratios of 340/380 nm.

[Ca²⁺]_{ER} was estimated by assessing the magnitude of the [Ca²⁺]_i responses induced by addition of 10 μ M thapsigargin (TG) (Thastrup et al. 1990). Eggs were maintained in Ca²⁺-free conditions, which were created by using TL-HEPES without adding CaCl₂ and supplemented with 1mM EGTA; TG was added to this media during [Ca²⁺]_i monitoring. [Ca²⁺]_i responses were then assessed by comparing the area-under-the-curve, which was calculated using the Prism software (GraphPad Software, La Jolla, CA).

IP₃ induced Ca²⁺ release was caused by caged-IP₃ (cIP₃). Eggs were first injected with 0.5 mM cIP₃ (Molecular Probes) and then loaded with 1.25 μ M Fluo-4 AM (Molecular Probes). IP₃ release was controlled by exposure to a 360 nm wavelength UV pulse. Fluo-4 excitation was accomplished with 488 nm wavelength and the emitted light collected as above; changes in [Ca²⁺]_i are expressed as $R = F/F_0$, where R is the fluorescence (F) normalized to the resting fluorescence (F₀). F₀ was calculated by averaging the first five fluorescent measurements for each egg prior to any treatment.

Western blotting

Cell lysates from 40 cumulus-free eggs were prepared by adding 15 μ l of 2X sample buffer (SB) (Laemmli 1970), as described previously (Jellerette et al. 2004). Samples were boiled for 3 min, loaded onto NuPAGE Novex 3–8% Tris-Acetate gels (Invitrogen, Carlsbad, CA), proteins were separated using electrophoresis and transferred onto nitrocellulose membranes (Micron Separations, Westboro, MA). IP₃R1 phosphorylation was probed with the MPM-2 antibody (1/500) (Upstate, Lake Placid, NY) and detection was accomplished by addition of a secondary HRP-conjugated goat anti-mouse antibody and chemiluminescence technology (NEN Life Science Products, Boston, MA). Blots were digitally recorded using a Kodak 440

Image Station (Rochester, NY). The same membranes were stripped at 50°C for 30 min (62.5 mM Tris, 2% SDS and 100 mM 2-beta mercaptoethanol) and were then used to detect IP₃R1 and its cleavage product. The Rbt03 antibody (1/1000; a generous gift of Dr J.B. Parys, Katholieke Universiteit, Leuven, Belgium) (Parys et al. 1995) was used to detect IP₃R1 followed by a HRP-labeled secondary antibody. The signal was captured as above, and quantification of the digital images was performed using Image J software (NIH) as previously described in our laboratory (Malcuit et al. 2005), although the absolute values of mean pixel intensity are graphed.

Immunofluorescence

Following removal of the zona pellucida with acid tyrode's solution (pH 2.7) and after washes in 0.1% BSA-supplemented Dulbecco's PBS (DPBS-BSA), eggs were first fixed in 3.7% paraformaldehyde supplemented with 0.02% Triton X-100 and subsequently permeabilized with 0.1% Triton X-100 supplemented DPBS-BSA. Eggs were transferred into DPBS+5% normal goat serum (NGS-DPBS) for 2 hrs at 4 °C followed by overnight incubation at 4 °C with an anti-IP₃R1 primary antibody (CT1; 1/100; a generous gift of Dr R.J. Wojcikiewicz, SUNY Upstate Medical University) (Wojcikiewicz et al. 1994). Following washing of the primary antibody, eggs were incubated with Alexa fluor 555-conjugated goat anti-rabbit IgG (Molecular Probes, Eugene, OR) for 1 hr at RT. Eggs were counterstained with 5 µg/ml Hoechst 33258 or with 1 µM TO-PRO-3 iodide (Invitrogen, Carlsbad, CA) and then mounted using Vectashield Mounting Media (Vector Laboratories, Burlingame, CA, USA). Slides were examined at RT with a confocal laser-scanning microscope (510 META, Carl Zeiss Microimaging, Inc., Germany) using an Axiovert 2 microscope outfitted with a 63 × 1.4 NA oil immersion objective lens. Z-stack images were obtained from cortical to equatorial planes every 2 to 5 µm. To visualize F-actin in living oocytes, 12.5 µM Alexa Fluor 546-conjugated phalloidin was injected into eggs. After injection, eggs were incubated for 30 min, after which images were captured using confocal microscope as described above. Phalloidin was prepared in methanol and stored at -20°C. On the day of the experiment, methanol was allowed to evaporate and DMSO was used to dissolve phalloidin to the desired concentration.

Mitochondria fluorescence

The membrane potential sensitive dye JC-1 (Molecular Probes, Eugene, Oregon, USA) was used to estimate the functional activity of mitochondria (Reers et al. 1995; Reers et al. 1991). A 2 mM stock solution was prepared in DMSO and kept at -20°. Before use, the stock solution was diluted to 2 µM in KSOM. Eggs were incubated with the working solution for 30 min at 37° before confocal observation. All eggs were captured under the same exposure intensity. In order to calculate the confocal ratio of red to green images, the intensity of green and red fluorescence were quantified separately with image-J software. Image J was also used to compare the size of red aggregates among groups: single aggregate was circled using the "elliptical selection tool", and then the area of the aggregate was measured in µm².

Parthenogenetic egg activation

Fresh, aged and caffeine-treated aged eggs were washed and incubated in Ca²⁺-free TL-hepes supplemented with 2 µM ionomycin (Sigma) for 5 min at room temperature. Eggs were then kept in cyloheximide (CHX, 20 µg/ml) only or CHX (10 µg/ml) + cytochalasin B (Cyto B, 5 µg/ml) in KSOM (0.1% PVA) at 36.5°C and in a humidified atmosphere containing 5% CO₂ for 4 hrs. After washing, eggs were transferred to KSOM medium and evaluated under a phase contrast microscopy for signs of activation 4 hrs after. Eggs beyond the MII stage were defined as activated and divided into two groups according to their appearance (1) the PN group, consisted of zygotes forming a single female PN with first and

second polar bodies; (2) the 2-cell group, consisted of zygotes that underwent immediate cleavage to 2-cells. Eggs without 2nd polar body, PN formation or 2-cells were defined as non-activated. Eggs that showed a fragmented cellular body were defined as fragmented.

Statistical analysis

Values from three or more experiments, performed on different batches of eggs, were used for evaluation of statistical significance. The Prism software (Graphpad Software) was used to draw graphs and perform the statistical comparisons using when appropriate Student's t-test or one-way ANOVA. Values are shown as means±S.E.M, and significant differences were considered at *p* values <0.05.

Acknowledgments

Grant sponsor: NIH; Grant number: R01 HD051872

The authors acknowledge the generous technical support and advice of Dr. Sook-Young Yoon for studies involving immunofluorescence and confocal microscopy. We thank Ms. Chang Li He for preparation of reagents. We also thank Hayley Yaglom and Robert Agreda for reading the manuscript and proving helpful suggestions.

Abbreviation

ART	assisted reproductive technologies
[Ca²⁺]_{ER}	calcium in ER
[Ca²⁺]_i	intracellular calcium
CHX	cycloheximide
cIP₃	caged-IP ₃
Cyto B	cytochalasin B
Δϕm	mitochondria membrane potential
DPBS	Dulbecco's PBS
DTT	DL-dithiothreitol
ER	endoplasmic reticulum
FCS	fetal calf serum
GSH	glutathione
hCG	human chorionic gonadotropin
Iono	Ionomycin
Hrs	hours
IC	immediate cleavage
ICSI	intracytoplasmic sperm injection
IP₃	inositol-1,4,5-trisphosphate
IP₃R1	inositol 1,4,5-trisphosphate receptor type 1
IVF	<i>in vitro</i> fertilization
KSOM	Potassium Simplex Optimized Medium
MAPK	Mitogen-activating protein kinase

MII	metaphase II
MPF	Mature promoting factor
NGS	normal goat serum
PIP₂	phosphatidylinositol 4,5-bisphosphate
PLCζ	sperm-specific phospholipase C
PMSG	pregnant mare serum gonadotropin
PN	pronucleus
PVA	polyvinyl alcohol
ROS	reactive oxygen species
RT	room temperature
SB	sample buffer
TG	thapsigargin
TL-HEPES	HEPES-buffered tyrode-lactate solution

References

- Abbott AL, Fissore RA, Ducibella T. Identification of a translocation deficiency in cortical granule secretion in preovulatory mouse oocytes. *Biol Reprod.* 2001; 65(6):1640–1647. [PubMed: 11717123]
- Abbott AL, Xu Z, Kopf GS, Ducibella T, Schultz RM. In vitro culture retards spontaneous activation of cell cycle progression and cortical granule exocytosis that normally occur in in vivo unfertilized mouse eggs. *Biology of Reproduction.* 1998; 59(6):1515–1521. [PubMed: 9828200]
- Azam S, Hadi N, Khan NU, Hadi SM. Antioxidant and prooxidant properties of caffeine, theobromine and xanthine. *Med Sci Monit.* 2003; 9(9):BR325–330. [PubMed: 12960921]
- Badenas J, Santalo J, Calafell JM, Estop AM, Egozcue J. Effect of the degree of maturation of mouse oocytes at fertilization: a source of chromosome imbalance. *Gamete Res.* 1989; 24(2):205–218. [PubMed: 2793059]
- Boerjan ML, de Boer P. First cell cycle of zygotes of the mouse derived from oocytes aged postovulation in vivo and fertilized in vivo. *Mol Reprod Dev.* 1990; 25(2):155–163. [PubMed: 2178641]
- Chi MM, Manchester JK, Yang VC, Curato AD, Strickler RC, Lowry OH. Contrast in levels of metabolic enzymes in human and mouse ova. *Biol Reprod.* 1988; 39(2):295–307. [PubMed: 2902884]
- Connors SA, Kanatsu-Shinohara M, Schultz RM, Kopf GS. Involvement of the cytoskeleton in the movement of cortical granules during oocyte maturation, and cortical granule anchoring in mouse eggs. *Dev Biol.* 1998; 200(1):103–115. [PubMed: 9698460]
- Ducibella T, Fissore R. The roles of Ca²⁺, downstream protein kinases, and oscillatory signaling in regulating fertilization and the activation of development. *Dev Biol.* 2008; 315(2):257–279. [PubMed: 18255053]
- Ducibella T, Schultz RM, Ozil JP. Role of calcium signals in early development. *Semin Cell Dev Biol.* 2006; 17(2):324–332. [PubMed: 16580237]
- Dumollard R, Marangos P, Fitzharris G, Swann K, Duchen M, Carroll J. Sperm-triggered [Ca²⁺] oscillations and Ca²⁺ homeostasis in the mouse egg have an absolute requirement for mitochondrial ATP production. *Development.* 2004; 131(13):3057–3067. [PubMed: 15163630]
- Eichenlaub-Ritter U, Stahl A, Luciani JM. The microtubular cytoskeleton and chromosomes of unfertilized human oocytes aged in vitro. *Hum Genet.* 1988; 80(3):259–264. [PubMed: 3192214]

- Essayan DM. Cyclic nucleotide phosphodiesterases. *J Allergy Clin Immunol.* 2001; 108(5):671–680. [PubMed: 11692087]
- Fissore RA, Kurokawa M, Knott J, Zhang M, Smyth J. Mechanisms underlying oocyte activation and postovulatory ageing. *Reproduction.* 2002; 124(6):745–754. [PubMed: 12530912]
- Fissore RA, Longo FJ, Anderson E, Parys JB, Ducibella T. Differential distribution of inositol trisphosphate receptor isoforms in mouse oocytes. *Biol Reprod.* 1999; 60(1):49–57. [PubMed: 9858485]
- FitzHarris G, Marangos P, Carroll J. Changes in endoplasmic reticulum structure during mouse oocyte maturation are controlled by the cytoskeleton and cytoplasmic dynein. *Dev Biol.* 2007; 305(1): 133–144. [PubMed: 17368610]
- Fukuda A, Roudabush WE, Thatcher SS. Influences of in vitro oocyte aging on microfertilization in the mouse with reference to zona hardening. *J Assist Reprod Genet.* 1992; 9(4):378–383. [PubMed: 1472818]
- Gordo AC, Rodrigues P, Kurokawa M, Jellerette T, Exley GE, Warner C, Fissore R. Intracellular calcium oscillations signal apoptosis rather than activation in in vitro aged mouse eggs. *Biol Reprod.* 2002; 66(6):1828–1837. [PubMed: 12021069]
- Goud AP, Goud PT, Diamond MP, Abu-Soud HM. Nitric Oxide Delays Oocyte Aging†. *Biochemistry.* 2005; 44(34):11361–11368. [PubMed: 16114873]
- Goud AP, Goud PT, Diamond MP, Gonik B, Abu-Soud HM. Reactive oxygen species and oocyte aging: role of superoxide, hydrogen peroxide, and hypochlorous acid. *Free Radic Biol Med.* 2008; 44(7):1295–1304. [PubMed: 18177745]
- Hirota J, Furuichi T, Mikoshiba K. Inositol 1,4,5-Trisphosphate Receptor Type 1 Is a Substrate for Caspase-3 and Is Cleaved during Apoptosis in a Caspase-3-dependent Manner. *Journal of Biological Chemistry.* 1999; 274(48):34433–34437. [PubMed: 10567423]
- Horne JH, Meyer T. Luminal calcium regulates the inositol trisphosphate receptor of rat basophilic leukemia cells at a cytosolic site. *Biochemistry.* 1995; 34(39):12738–12746. [PubMed: 7548027]
- Igarashi H, Takahashi E, Hiroi M, Doi K. Aging-related changes in calcium oscillations in fertilized mouse oocytes. *Mol Reprod Dev.* 1997; 48(3):383–390. [PubMed: 9322251]
- Igarashi H, Takahashi T, Takahashi E, Tezuka N, Nakahara K, Takahashi K, Kurachi H. Aged mouse oocytes fail to readjust intracellular adenosine triphosphates at fertilization. *Biol Reprod.* 2005; 72(5):1256–1261. [PubMed: 15659708]
- Iwamoto M, Onishi A, Fuchimoto D, Somfai T, Suzuki S, Yazaki S, Hashimoto M, Takeda K, Tagami T, Hanada H, Noguchi J, Kaneko H, Nagai T, Kikuchi K. Effects of caffeine treatment on aged porcine oocytes: parthenogenetic activation ability, chromosome condensation and development to the blastocyst stage after somatic cell nuclear transfer. *Zygote.* 2005; 13(4):335–345. [PubMed: 16388702]
- Jellerette T, Kurokawa M, Lee B, Malcuit C, Yoon SY, Smyth J, Vermassen E, De Smedt H, Parys JB, Fissore RA. Cell cycle-coupled $[Ca^{2+}]_i$ oscillations in mouse zygotes and function of the inositol 1,4,5-trisphosphate receptor-1. *Dev Biol.* 2004; 274(1):94–109. [PubMed: 15355791]
- Jones KT, Carroll J, Whittingham DG. Ionomycin, thapsigargin, ryanodine, and sperm induced Ca^{2+} release increase during meiotic maturation of mouse oocytes. *J Biol Chem.* 1995; 270(12):6671–6677. [PubMed: 7896808]
- Jones KT, Whittingham DG. A comparison of sperm- and IP_3 -induced Ca^{2+} release in activated and aging mouse oocytes. *Dev Biol.* 1996; 178(2):229–237. [PubMed: 8812125]
- Joseph SK, Hajnoczky G. IP_3 receptors in cell survival and apoptosis: Ca^{2+} release and beyond. *Apoptosis.* 2007; 12(5):951–968. [PubMed: 17294082]
- Kamat JP, Bloor KK, Devasagayam TP, Jayashree B, Kesavan PC. Differential modification by caffeine of oxygen-dependent and independent effects of gamma-irradiation on rat liver mitochondria. *Int J Radiat Biol.* 2000; 76(9):1281–1288. [PubMed: 10993639]
- Kaufman MH, Sachs L. Complete preimplantation development in culture of parthenogenetic mouse embryos. *Journal of embryology and experimental morphology.* 1976; 35(1):179–190. [PubMed: 1270978]

- Kesavan PC, Powers EL. Differential modification of oxic and anoxic components of radiation damage in *Bacillus megaterium* spores by caffeine. *Int J Radiat Biol Relat Stud Phys Chem Med*. 1985; 48(2):223–233. [PubMed: 3926682]
- Khan MT, Bhanumathy CD, Schug ZT, Joseph SK. Role of inositol 1,4,5-trisphosphate receptors in apoptosis in DT40 lymphocytes. *J Biol Chem*. 2007; 282(45):32983–32990. [PubMed: 17875645]
- Kikuchi K, Izaike Y, Noguchi J, Furukawa T, Daen FP, Naito K, Toyoda Y. Decrease of histone H1 kinase activity in relation to parthenogenetic activation of pig follicular oocytes matured and aged in vitro. *J Reprod Fertil*. 1995; 105(2):325–330. [PubMed: 8568778]
- Kikuchi K, Naito K, Noguchi J, Kaneko H, Tojo H. Maturation/M-phase promoting factor regulates aging of porcine oocytes matured in vitro. *Cloning Stem Cells*. 2002; 4(3):211–222. [PubMed: 12398802]
- Kikuchi K, Naito K, Noguchi J, Shimada A, Kaneko H, Yamashita M, Aoki F, Tojo H, Toyoda Y. Maturation/M-phase promoting factor: a regulator of aging in porcine oocytes. *Biol Reprod*. 2000; 63(3):715–722. [PubMed: 10952912]
- Kikuchi K, Naito K, Noguchi J, Shimada A, Kaneko H, Yamashita M, Tojo H, Toyoda Y. Inactivation of p34cdc2 kinase by the accumulation of its phosphorylated forms in porcine oocytes matured and aged in vitro. *Zygote*. 1999; 7(2):173–179. [PubMed: 10418112]
- Kim NH, Moon SJ, Prather RS, Day BN. Cytoskeletal alteration in aged porcine oocytes and parthenogenesis. *Mol Reprod Dev*. 1996; 43(4):513–518. [PubMed: 9052943]
- Kline D. Attributes and dynamics of the endoplasmic reticulum in mammalian eggs. *Curr Top Dev Biol*. 2000; 50:125–154. [PubMed: 10948453]
- Kothakota S, Azuma T, Reinhard C, Klippel A, Tang J, Chu K, McGarry TJ, Kirschner MW, Kohts K, Kwiatkowski DJ, Williams LT. Caspase-3-generated fragment of gelsolin: effector of morphological change in apoptosis. *Science*. 1997; 278(5336):294–298. [PubMed: 9323209]
- Kurokawa M, Fissore RA. ICSI-generated mouse zygotes exhibit altered calcium oscillations, inositol 1,4,5-trisphosphate receptor-1 down-regulation, and embryo development. *Mol Hum Reprod*. 2003; 9(9):523–533. [PubMed: 12900511]
- Kurokawa M, Sato K-i, Smyth J, Wu H, Fukami K, Takenawa T, Fissore RA. Evidence that activation of Src family kinase is not required for fertilization-associated $[Ca^{2+}]_i$ oscillations in mouse eggs. *Reproduction*. 2004; 127(4):441–454. [PubMed: 15047935]
- Kwon DJ, Park CK, Yang BK, Cheong HT. Control of nuclear remodelling and subsequent in vitro development and methylation status of porcine nuclear transfer embryos. *Reproduction*. 2008; 135(5):649–656. [PubMed: 18411411]
- Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*. 1970; 227(5259):680–685. [PubMed: 5432063]
- Lee B, Vermassen E, Yoon SY, Vanderheyden V, Ito J, Alfandari D, De Smedt H, Parys JB, Fissore RA. Phosphorylation of IP3R1 and the regulation of $[Ca^{2+}]_i$ responses at fertilization: a role for the MAP kinase pathway. *Development*. 2006; 133(21):4355–4365. [PubMed: 17038520]
- Lee JH, Campbell KH. Caffeine treatment prevents age-related changes in ovine oocytes and increases cell numbers in blastocysts produced by somatic cell nuclear transfer. *Cloning Stem Cells*. 2008; 10(3):381–390. [PubMed: 18673075]
- Liu L, Hammar K, Smith PJ, Inoue S, Keefe DL. Mitochondrial modulation of calcium signaling at the initiation of development. *Cell Calcium*. 2001; 30(6):423–433. [PubMed: 11728137]
- Maalouf WE, Lee JH, Campbell KH. Effects of caffeine, cumulus cell removal and aging on polyspermy and embryo development on in vitro matured and fertilized ovine oocytes. *Theriogenology*. 2009; 71(7):1083–1092. [PubMed: 19185338]
- Mailhes JB, Young D, London SN. Postovulatory ageing of mouse oocytes in vivo and premature centromere separation and aneuploidy. *Biol Reprod*. 1998; 58(5):1206–1210. [PubMed: 9603254]
- Malcuit C, Knott JG, He C, Wainwright T, Parys JB, Robl JM, Fissore RA. Fertilization and inositol 1,4,5-trisphosphate (IP3)-induced calcium release in type-1 inositol 1,4,5-trisphosphate receptor down-regulated bovine eggs. *Biol Reprod*. 2005; 73(1):2–13. [PubMed: 15744020]
- Manonmani P, Okada H, Ogqnuqi N, Uda A, Ogura A, Yoshida T, Sankai T. Fertilization and preimplantation development of mouse oocytes after prolonged incubation with caffeine. *Reproductive Medicine and Biology*. 2004; 3(4):245–251.

- Marston JH, Chang MC. The Fertilizable Life of Ova and Their Morphology Following Delayed Insemination in Mature and Immature Mice. *J Exp Zool.* 1964; 155:237–251. [PubMed: 14131458]
- Mehlmann LM, Mikoshiba K, Kline D. Redistribution and increase in cortical inositol 1,4,5-trisphosphate receptors after meiotic maturation of the mouse oocyte. *Dev Biol.* 1996; 180(2):489–498. [PubMed: 8954721]
- Miao YL, Kikuchi K, Sun QY, Schatten H. Oocyte aging: cellular and molecular changes, developmental potential and reversal possibility. *Hum Reprod Update.* 2009; 15(5):573–585. [PubMed: 19429634]
- Missiaen L, Taylor CW, Berridge MJ. Spontaneous calcium release from inositol trisphosphate-sensitive calcium stores. *Nature.* 1991; 352(6332):241–244. [PubMed: 1857419]
- Miyazaki S. Thirty years of calcium signals at fertilization. *Seminars in Cell & Developmental Biology.* 2006; 17(2):233–243. [PubMed: 16549376]
- Mukhopadhyay S, Mondal A, Poddar MK. Chronic administration of caffeine: effect on the activities of hepatic antioxidant enzymes of Ehrlich ascites tumor-bearing mice. *Indian J Exp Biol.* 2003; 41(4):283–289. [PubMed: 15255635]
- Oldershaw KA, Taylor CW. Luminal Ca^{2+} increases the affinity of inositol 1,4,5-trisphosphate for its receptor. *Biochem J.* 1993; 292 (Pt 3):631–633. [PubMed: 8391254]
- Ono T, Mizutani E, Li C, Yamagata K, Wakayama T. Offspring from intracytoplasmic sperm injection of aged mouse oocytes treated with caffeine or MG132. *Genesis.* 2011; 49(6):460–471. [PubMed: 21504043]
- Ozil JP, Banrezes B, Toth S, Pan H, Schultz RM. Ca^{2+} oscillatory pattern in fertilized mouse eggs affects gene expression and development to term. *Dev Biol.* 2006; 300(2):534–544. [PubMed: 16996050]
- Ozil JP, Markoulaki S, Toth S, Matson S, Banrezes B, Knott JG, Schultz RM, Huneau D, Ducibella T. Egg activation events are regulated by the duration of a sustained $[\text{Ca}^{2+}]_{\text{cyt}}$ signal in the mouse. *Dev Biol.* 2005; 282(1):39–54. [PubMed: 15936328]
- Papandile A, Tyas D, O'Malley DM, Warner CM. Analysis of caspase-3, caspase-8 and caspase-9 enzymatic activities in mouse oocytes and zygotes. *Zygote.* 2004; 12(1):57–64. [PubMed: 15214581]
- Parrington J, Brind S, De Smedt H, Gangeswaran R, Lai FA, Wojcikiewicz R, Carroll J. Expression of inositol 1,4,5-trisphosphate receptors in mouse oocytes and early embryos: the type I isoform is upregulated in oocytes and downregulated after fertilization. *Dev Biol.* 1998; 203(2):451–461. [PubMed: 9808793]
- Parys JB, de Smedt H, Missiaen L, Bootman MD, Sienaert I, Casteels R. Rat basophilic leukemia cells as model system for inositol 1,4,5-trisphosphate receptor IV, a receptor of the type II family: functional comparison and immunological detection. *Cell Calcium.* 1995; 17(4):239–249. [PubMed: 7664312]
- Perez GI, Tao XJ, Tilly JL. Fragmentation and death (a.k.a. apoptosis) of ovulated oocytes. *Mol Hum Reprod.* 1999; 5(5):414–420. [PubMed: 10338364]
- Rausell F, Pertusa JF, Gomez-Piquer V, Hermenegildo C, Garcia-Perez MA, Cano A, Tarin JJ. Beneficial effects of dithiothreitol on relative levels of glutathione S-transferase activity and thiols in oocytes, and cell number, DNA fragmentation and allocation at the blastocyst stage in the mouse. *Mol Reprod Dev.* 2007; 74(7):860–869. [PubMed: 17186547]
- Reers M, Smiley ST, Mottola-Hartshorn C, Chen A, Lin M, Chen LB. Mitochondrial membrane potential monitored by JC-1 dye. *Methods in enzymology.* 1995; 260:406–417. [PubMed: 8592463]
- Reers M, Smith TW, Chen LB. J-aggregate formation of a carbocyanine as a quantitative fluorescent indicator of membrane potential. *Biochemistry.* 1991; 30(18):4480–4486. [PubMed: 2021638]
- Rodman TC. Chromatid disjunction in unfertilized ageing oocytes. *Nature.* 1971; 233(5316):191–193. [PubMed: 4106820]
- Roede JR, Jones DP. Reactive species and mitochondrial dysfunction: mechanistic significance of 4-hydroxynonenal. *Environ Mol Mutagen.* 2010; 51(5):380–390. [PubMed: 20544880]

- Sablina AA, Chumakov PM, Levine AJ, Kopnin BP. p53 activation in response to microtubule disruption is mediated by integrin-Erk signaling. *Oncogene*. 2001; 20(8):899–909. [PubMed: 11314025]
- Sakai N, Endo A. Effects of delayed mating on preimplantation embryos in spontaneously ovulated mice. *Gamete Res*. 1988; 19(4):381–385. [PubMed: 3198058]
- Saunders CM, Larman MG, Parrington J, Cox LJ, Royse J, Blayney LM, Swann K, Lai FA. PLC zeta: a sperm-specific trigger of Ca(2+) oscillations in eggs and embryo development. *Development*. 2002; 129(15):3533–3544. [PubMed: 12117804]
- Sienaert I, Missiaen L, De Smedt H, Parys JB, Sipma H, Casteels R. Molecular and functional evidence for multiple Ca²⁺-binding domains in the type 1 inositol 1,4,5-trisphosphate receptor. *J Biol Chem*. 1997; 272(41):25899–25906. [PubMed: 9325322]
- Smith AL, Lodge JR. Interactions of aged gametes: in vitro fertilization using in vitro-aged sperm and in vivo-aged ova in the mouse. *Gamete Res*. 1987; 16(1):47–56. [PubMed: 3506899]
- Steuerwald NM, Steuerwald MD, Mailhes JB. Post-ovulatory aging of mouse oocytes leads to decreased MAD2 transcripts and increased frequencies of premature centromere separation and anaphase. *Mol Hum Reprod*. 2005; 11(9):623–630. [PubMed: 16207798]
- Stricker SA. Comparative biology of calcium signaling during fertilization and egg activation in animals. *Dev Biol*. 1999; 211(2):157–176. [PubMed: 10395780]
- Szollosi D. Morphological changes in mouse eggs due to aging in the fallopian tube. *Am J Anat*. 1971; 130(2):209–225. [PubMed: 5547003]
- Takahashi T, Igarashi H, Kawagoe J, Amita M, Hara S, Kurachi H. Poor Embryo Development in Mouse Oocytes Aged In Vitro Is Associated with Impaired Calcium Homeostasis. *Biol Reprod*. 2009; 80(3):493–502. [PubMed: 19038861]
- Takahashi T, Saito H, Hiroi M, Doi K, Takahashi E. Effects of aging on inositol 1,4,5-triphosphate-induced Ca(2+) release in unfertilized mouse oocytes. *Mol Reprod Dev*. 2000; 55(3):299–306. [PubMed: 10657049]
- Tarin JJ. Potential effects of age-associated oxidative stress on mammalian oocytes/embryos. *Mol Hum Reprod*. 1996; 2(10):717–724. [PubMed: 9239688]
- Tarin JJ, Perez-Albala S, Aguilar A, Minarro J, Hermenegildo C, Cano A. Long-term effects of postovulatory aging of mouse oocytes on offspring: a two-generational study. *Biol Reprod*. 1999; 61(5):1347–1355. [PubMed: 10529284]
- Tarin JJ, Perez-Albala S, Cano A. Consequences on offspring of abnormal function in ageing gametes. *Hum Reprod Update*. 2000; 6(6):532–549. [PubMed: 11129687]
- Tarin JJ, Ten J, Vendrell FJ, Cano A. Dithiothreitol prevents age-associated decrease in oocyte/conceptus viability in vitro. *Hum Reprod*. 1998; 13(2):381–386. [PubMed: 9557843]
- Tatone C, Carbone MC, Gallo R, Delle Monache S, Di Cola M, Alesse E, Amicarelli F. Age-associated changes in mouse oocytes during postovulatory in vitro culture: possible role for meiotic kinases and survival factor BCL2. *Biol Reprod*. 2006; 74(2):395–402. [PubMed: 16251501]
- Thastrup O, Cullen PJ, Drobak BK, Hanley MR, Dawson AP. Thapsigargin, a tumor promoter, discharges intracellular Ca²⁺ stores by specific inhibition of the endoplasmic reticulum Ca²⁺(+)-ATPase. *Proc Natl Acad Sci U S A*. 1990; 87(7):2466–2470. [PubMed: 2138778]
- Thouas GA, Trounson AO, Wolvetang EJ, Jones GM. Mitochondrial dysfunction in mouse oocytes results in preimplantation embryo arrest in vitro. *Biol Reprod*. 2004; 71(6):1936–1942. [PubMed: 15286028]
- Trounson AO, Mohr LR, Wood C, Leeton JF. Effect of delayed insemination on in-vitro fertilization, culture and transfer of human embryos. *J Reprod Fertil*. 1982; 64(2):285–294. [PubMed: 7069652]
- Van Blerkom J, Davis P, Mathwig V, Alexander S. Domains of high-polarized and low-polarized mitochondria may occur in mouse and human oocytes and early embryos. *Hum Reprod*. 2002; 17(2):393–406. [PubMed: 11821285]
- Van Wissen B, Bomsel-Helmreich O, Debey P, Eisenberg C, Vautier D, Pennehouat G. Fertilization and ageing processes in non-divided human oocytes after GnRHa treatment: an analysis of individual oocytes. *Hum Reprod*. 1991; 6(6):879–884. [PubMed: 1757529]

- Verbert L, Lee B, Kocks SL, Assefa Z, Parys JB, Missiaen L, Callewaert G, Fissore RA, De Smedt H, Bultynck G. Caspase-3-truncated type 1 inositol 1,4,5-trisphosphate receptor enhances intracellular Ca^{2+} leak and disturbs Ca^{2+} signalling. *Biol Cell*. 2008; 100(1):39–49. [PubMed: 17868032]
- Wang LY, Wang DH, Zou XY, Xu CM. Mitochondrial functions on oocytes and preimplantation embryos. *J Zhejiang Univ Sci B*. 2009; 10(7):483–492. [PubMed: 19585665]
- Webb M, Howlett SK, Maro B. Parthenogenesis and cytoskeletal organization in ageing mouse eggs. *J Embryol Exp Morphol*. 1986; 95(1):131–145. [PubMed: 3794588]
- Wessel GM, Conner SD, Berg L. Cortical granule translocation is microfilament mediated and linked to meiotic maturation in the sea urchin oocyte. *Development*. 2002; 129(18):4315–4325. [PubMed: 12183383]
- Wilcox AJ, Weinberg CR, Baird DD. Post-ovulatory ageing of the human oocyte and embryo failure. *Hum Reprod*. 1998; 13(2):394–397. [PubMed: 9557845]
- Wilding M, Dale B, Marino M, di Matteo L, Alviggi C, Pisaturo ML, Lombardi L, De Placido G. Mitochondrial aggregation patterns and activity in human oocytes and preimplantation embryos. *Hum Reprod*. 2001; 16(5):909–917. [PubMed: 11331637]
- Wojcikiewicz RJ, Furuichi T, Nakade S, Mikoshiba K, Nahorski SR. Muscarinic receptor activation down-regulates the type I inositol 1,4,5-trisphosphate receptor by accelerating its degradation. *J Biol Chem*. 1994; 269(11):7963–7969. [PubMed: 8132516]
- Xu Z, Abbott A, Kopf GS, Schultz RM, Ducibella T. Spontaneous activation of ovulated mouse eggs: time-dependent effects on M-phase exit, cortical granule exocytosis, maternal messenger ribonucleic acid recruitment, and inositol 1,4,5-trisphosphate sensitivity. *Biology of Reproduction*. 1997; 57(4):743–750. [PubMed: 9314575]
- Xu Z, Williams CJ, Kopf GS, Schultz RM. Maturation-associated increase in IP3 receptor type 1: role in conferring increased IP3 sensitivity and Ca^{2+} oscillatory behavior in mouse eggs. *Dev Biol*. 2003; 254(2):163–171. [PubMed: 12591238]
- Yanagida K, Yazawa H, Katayose H, Suzuki K, Hoshi K, Sato A. Influence of oocyte preincubation time on fertilization after intracytoplasmic sperm injection. *Hum Reprod*. 1998; 13(8):2223–2226. [PubMed: 9756300]
- Yanagimachi R, Chang MC. Fertilizable life of golden hamster ova and their morphological changes at the time of losing fertilizability. *J Exp Zool*. 1961; 148:185–203. [PubMed: 14008938]
- Zackowski JL, Leon PAM-D. Second Meiotic Nondisjunction Is Not Increased in Postovulatory Aged Murine Oocytes Fertilized in vitro. *In Vitro Cellular & Developmental Biology*. 1988; 24(2): 133–137. [PubMed: 3343191]

Figure 1.

Post-ovulatory aging of eggs reduces IP_3 induced Ca^{2+} release.

(A): Changes in $[Ca^{2+}]_i$ induced by photolysis of cIP_3 in fresh (14hrs post hCG) and *in vitro* aged (38hrs post hCG) mouse eggs. Duration of UV pulse is noted in each of the panels. (B): Quantification of $[Ca^{2+}]_i$ rise parameters corresponding to each UV pulse are shown on bar graphs to the right of the $[Ca^{2+}]_i$ tracings. Values are means $\pm$ SEM, and bars with 2 asterisks are different those without them with $p < 0.01$ here and throughout the manuscript.

Figure 2.

$[Ca^{2+}]_{ER}$ is decreased in aged eggs.

(A): Representative $[Ca^{2+}]_i$ profiles of Ca^{2+} release from the ER induced by addition of TG in fresh and *in vitro* aged mouse eggs. (B): Area under the curve for TG-induced Ca^{2+} release was calculated for both MII and *in vitro* aged mouse eggs; MII was chosen as 100% and values for aged eggs are presented relative to 100%. The presence of an asterisk denotes treatments that are different at $p < 0.05$ here and all figures.

Figure 3.

IP₃R1 sensitivity decreases in aged eggs.

(A): Representative traces of $[Ca^{2+}]_i$ changes induced by a threshold cIP₃ pulse in fresh and *in vitro* aged mouse eggs. (B): Representative $[Ca^{2+}]_i$ changes induced by a saturating pulse of cIP₃ in fresh and *in vitro* aged eggs. (C): Mean values of peak $[Ca^{2+}]_i$ rises induced by both duration cIP₃ pulses in fresh and aged eggs presented as a line graph. (D): Normalized values of peak $[Ca^{2+}]_i$ rise caused by both pulses of cIP₃; value for saturating cIP₃ pulse was chosen as 100% for each group. (E): Bar graph representation of the percentage of $[Ca^{2+}]_i$ release caused by the threshold cIP₃ pulse vs. total $[Ca^{2+}]_i$ release caused by saturating cIP₃ pulse in MII and aged eggs.

Figure 4.

IP₃R1 undergoes biochemical de-differentiation during egg aging.

(A): Immunoblotting of egg lysates from fresh, aged non-fragmented and aged-fragmented eggs probed with the Rbt03 antibody. Quantification of fragmented IP₃R1 band is shown in the bar graph to the right of the IB panel. Bands within the box represent the putative C-term IP₃R1 cleavage product (B): The same blots were probed with MPM-2 antibody.

Quantification of IP₃R1 MPM-2 reactivity is shown to the right of the IB panel. (C): Immunostaining of IP₃R1 distribution in fresh and *in vitro* aged mouse eggs shows loss of IP₃R1s cortical clusters organization in aged eggs and also a more diffuse staining in these cells. Filled arrow in left panel indicates IP₃R1 cortical clusters, while the empty arrow in right panel denotes the absence of such clusters in aged eggs.

Figure 5.

Caffeine prevents the decrease in IP₃ mediated Ca²⁺ release and rescue the loss of IP₃R_{1S} sensitivity associated with eggs aging.

(A): Traces of [Ca²⁺]_i changes caused by photolysis of cIP₃ in fresh, *in vitro* aged, *in vitro* aged+100 ng/ml colcemid, and *in vitro* aged+5mM caffeine mouse eggs. Duration of UV pulses is noted in the [Ca²⁺]_i panels. Quantifications and statistical comparison of the induced [Ca²⁺]_i responses are to right of the [Ca²⁺]_i traces. **(B):** Traces of [Ca²⁺]_i changes after a near threshold pulse of cIP₃ in fresh, *in vitro* aged, and *in vitro* aged+5mM caffeine mouse eggs. **(C):** [Ca²⁺]_i changes after a saturating release of cIP₃ in fresh, *in vitro* aged, and *in vitro* aged+5mM caffeine mouse eggs. **(D):** Normalized value of peak [Ca²⁺]_i caused by both pulses of cIP₃ in fresh, *in vitro* aged and *in vitro* aged+5mM caffeine mouse eggs; value for saturating cIP₃ pulse was used as a 100% response for each group.

Figure 6.

Exposure to caffeine maintains $[Ca^{2+}]_i$ oscillatory responses in aged eggs and prevents the decrease in $[Ca^{2+}]_{ER}$ associated with egg aging.

(A): Changes in $[Ca^{2+}]_i$ induced by microinjection of PLC ζ mRNA (0.05 μ g/ μ l) in fresh, *in vitro* aged, *in vitro* aged+100 ng/ml colcemid, and *in vitro* aged+5mM caffeine mouse eggs. Fresh MII and caffeine-treated eggs initiated responses earlier than the other groups and with significant differences in the duration of the first $[Ca^{2+}]_i$ rise. **(B):** Bar graph showing statistical comparisons of the duration of 1st $[Ca^{2+}]_i$ rise obtained in figure A. **(C):** Release of Ca^{2+} from ER by addition of TG in fresh, *in vitro* aged, and *in vitro* aged+5mM caffeine mouse eggs. **(D):** Quantifications and statistical comparison of the area under the curve of $[Ca^{2+}]_i$ responses in C. Bars with different superscripts represent treatments that are significantly different ($P < 0.05$).

Figure 7.

Caffeine alters IP₃R1 organization, actin distribution and mitochondria activity in aged eggs. **(A):** Immunostaining of IP₃R1s in fresh, *in vitro* aged, and *in vitro* aged+5mM caffeine treated mouse eggs. Filled arrow in left panel denotes IP₃R1 cortical clusters in fresh MII eggs, while the empty arrow in middle panel denotes absence of IP₃R1 cortical clusters in aged eggs, and empty arrow with broken lines in right panel denotes accumulation of IP₃R1 in large patches in caffeine-treated aged eggs. **(B):** Filled arrow in left and right panels indicates the presence of the actin cap overlaying the spindle area in fresh MII and in caffeine-treated aged eggs. The empty arrow in middle panel denotes the absence of the actin cap on the corresponding spindle area of aged eggs. **(C):** Changes in mitochondria membrane potential, as reported by JC-1 fluorescence (2μM), in fresh, *in vitro* aged, and *in vitro* aged+5mM caffeine. **(D):** Bar graph showing mean values of the quantification of the intensity ratio (red/green) of JC-1 staining in fresh, *in vitro* aged, and *in vitro* aged+5mM caffeine mouse eggs. **(E):** Comparison of the area of red JC-1 aggregates in fresh, *in vitro* aged, and *in vitro* aged+5mM caffeine mouse eggs. The 10 biggest aggregates were chosen for quantification, which was performed using Image J software. Data are the mean ± SEM. Bars with different superscripts represent treatments that are significantly different, P < 0.05.

A **Figure 8.**

Effects of caffeine treatment on activation and development ability of aged eggs.

(A): Representative activation outcomes of eggs observed 4 and 24 hrs after activation with Iono+CHX or Iono+CHX+Cyto B. While fresh eggs show normal signs of activation corresponding with the time of observation, aged eggs became mostly fragmented, and caffeine-treated aged eggs underwent immediate cleavage to the 2-cell stage.

Table 1
The effects of caffeine on development of mouse eggs induced by parthenogenetic activation without Cyto B

		Iono + CHX activation (%)							
		4hrs				24hrs			
		MII	PN	2-cell	Fragment	MII	PN	2-cell	Fragment
Fresh (n=116)		5.9±2.2 ^a	94.1±2.2 ^a	-	-	4.9±1.9 ^a	7.3±4.7	87.8±3.8 ^a	-
0mM caffeine (n=104)		42.1±8.9 ^b	2.3±1.9 ^b	-	55.6±8.9 ^a	4.0±1.4 ^a	-	-	96.0±1.4 ^a
5mM caffeine (n=111)		-	11.4±6.0 ^b	65.6±8.2	23.4±4.0 ^b	-	-	64.4±7.5 ^b	35.6±7.5 ^b

¹ Iono+CHX: Ionomycin (2.5 μM, 5mins) followed by cycloheximide (20 μg/ml, 4hrs).

² Data are the mean ±SEM.

^{3a-b} Values with different superscript letters within the same column are significantly different, P <0.05.

The effects of caffeine on development of mouse eggs induced by parthenogenetic activation with Cyto B

Table 2

Iono + CHX+ Cyto B activation (%)								
	4hrs				24hrs			
	MII	PN	2-cell	Fragment	MII	PN	2-cell	Fragment
Fresh (n=116)	23.3±6.2 ^a	76.6±6.2 ^a	-	-	11.0±4.0 ^a	18.4±6.7	70.6±3.4 ^a	-
0mM caffeine (n=110)	79.2±5.1 ^b	-	-	20.8±5.1 ^a	9.8±7.5 ^b	-	-	90.2±7.5 ^a
5mM caffeine (n=102)	76.7±3.2 ^b	7.1±2.1 ^b	-	17.2±5.2 ^a	-	-	71.9±8.4 ^a	28.1±8.4 ^b

¹ Iono+CHX+Cyto B: Ionomycin (2.5 μM, 5mins) followed by cycloheximide (10 μg/ml)+cytochalasin B (5 μg/ml, 4hrs).

² Data are the mean ±SEM.

³ *a-b* Values with different superscript letters within the same column are significantly different, P <0.05.