Membrane and cortical abnormalities in post-ovulatory aged eggs: analysis of fertilizability and establishment of the membrane block to polyspermy

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Fertilization at increased times after ovulation is associated with poor reproductive outcomes. This study examines the effects of post-ovulatory ageing on egg membrane function through analyses of mouse eggs collected at 13 and 22 h post-HCG (‘young’ and ‘aged’ eggs, respectively). Experiments in which fertilized zona pellucida-free young and aged eggs are challenged with additional sperm reveal that aged eggs are less able to establish a membrane block to prevent polyspermy, since sperm penetrate 24% of fertilized aged eggs but are unable to penetrate fertilized young eggs. This is not due to a failure of aged eggs to respond to fertilization, as the extent of sperm-induced cortical granule exocytosis is similar in aged and young eggs. Post-ovulatory ageing also affects egg membrane receptivity to sperm as a subset of zona pellucida-free aged eggs are slow to fertilize or resistant to fertilization. Sperm binding to young and aged eggs is similar, but aged eggs develop cytoskeletal abnormalities that may affect membrane/cortical function, such as the ability of the egg membrane to support sperm–egg fusion. These data demonstrate that the poor reproductive outcomes associated with post-ovulatory ageing could be a result of abnormalities that may affect membrane/cortical function, such as the ability of the egg membrane to support sperm–egg fusion. These data demonstrate that the poor reproductive outcomes associated with post-ovulatory ageing could be a result of reduced fertilization, due to reduced egg membrane receptivity to sperm, or a result of increased incidence of polyspermy, due to the reduced ability to establish a membrane block to polyspermy. This analysis of egg membrane function deficiencies provides insights into post-ovulatory ageing and has implications for assisted reproductive technologies.

Key words: actin/cytoskeleton/egg activation/polyspermy/post-ovulatory ageing

Introduction

There is evidence that reproductive success decreases if conception occurs at later times after ovulation. Problems associated with delayed conception include decreased litter size in animals and low pregnancy rates and an increased risk of spontaneous abortion in human (Blandau and Young, 1939; Blandau and Jordan, 1941; Guerrero and Lancaster, 1970; Guerrero and Rojas, 1975; Gray et al., 1995; Wilcox et al., 1998; Tarin et al., 2000). However, it is not known why these reproductive problems are associated with post-ovulatory ageing. A variety of studies raise the possibility of two, not mutually exclusive factors that could contribute to the reduced reproductive success with delayed conception (discussed below). The work presented here uses IVF studies to test specific hypotheses regarding the cellular consequences of eggs undergoing post-ovulatory ageing, using mouse as an experimental model system.

One possible factor contributing to the reduced reproductive success with delayed conception is reduced incidence of fertilization. Animal studies of natural mating or artificial insemination at increased times after ovulation report observations of sperm in the perivitelline space of unfertilized eggs, suggestive of reduced sperm penetration of the egg plasma membrane (Blandau, 1952; Austin and Braden, 1953a; Yanagimachi and Chang, 1961; Marston and Chang, 1964). However, a limitation of these in vivo studies is the difficulty in distinguishing between effects due to post-ovulatory ageing of the eggs, effects on the sperm in the female tract, and/or other reproductive tract changes that occur following ovulation. Reduced fertilization of aged mouse and human eggs inseminated in vitro has been reported (Wolf and Hamada, 1976; Park et al., 2000), although the cause(s) of this reduced fertilization is not known. Because of reports of perivitelline sperm in unfertilized aged eggs (Blandau, 1952; Austin and Braden, 1953a; Yanagimachi and Chang, 1961; Marston and Chang, 1964), here we test the hypothesis that the aged egg plasma membrane has reduced receptivity to sperm.

A second possible contributing factor to reduced reproductive success with delayed conception is an increased incidence of polyspermy, or fertilization of an egg by more than one sperm. Data from animal models suggest that the incidence of polyspermy increases with increased time after ovulation (Austin and Braden, 1953a,b; Odor and Blandau, 1956; Yanagimachi and Chang, 1961). The majority of triploid human and mouse embryos are the result of two sperm fertilizing an egg (Beatty, 1978; Jacobs et al., 1978; Uchida and Freeman, 1985; Santalo et al., 1986; Zaragoza et al., 2000), with most triploid embryos dying during early development [triploidy is detected in ~10% of spontaneously aborted human concepti (Jacobs et al., 1978; Hassold et al., 1980; Michelmann et al., 1986; Robinson et al., 2001; Stephenson et al., 2002)] and a
small subset surviving longer, up to the third trimester, or dying shortly after birth (Jacobs et al., 1978; Hassold et al., 1980).

An increased incidence of polyspermy in vivo at later times after ovulation (Austin and Braden, 1953a,b; Odor and Blandau, 1956; Yanagimachi and Chang, 1961) raises the possibility that fertilized aged eggs have an impaired ability to prevent polyspermic fertilization. Mammalian eggs use mechanisms, called blocks to polyspermy, to prevent fertilization by more than one sperm. The zona pellucida block to polyspermy is the result of cortical granule (CG) exocytosis, induced by the increase in cytosolic calcium following sperm–egg fusion; the release of CG contents converts the zona pellucida to a form that poorly supports sperm binding (Yanagimachi, 1994; Abbott and Ducibella, 2001). Less is known about the membrane block to polyspermy, although evidence for it comes from numerous studies (Austin, 1961; Wolf, 1978; Zuccotti et al., 1991; Horvath et al., 1993; Maluchnik and Borsuk, 1994; Sengoku et al., 1995; McAvey et al., 2002). In particular, experiments in which fertilized eggs are challenged with additional sperm reveal that zona pellucida-free fertilized mouse, hamster and human eggs are resistant to penetration by additional sperm, indicating that the egg membrane changes upon fertilization to reduce receptivity to sperm (Wolf, 1978; Zuccotti et al., 1991; Horvath et al., 1993; Maluchnik and Borsuk, 1994; Sengoku et al., 1995). Evidence for a membrane block in human eggs comes from IVF experiments of zona pellucida-free human eggs; results from these experiments precisely parallel the findings with other mammalian eggs (Sengoku et al., 1995). Interestingly, polyspermy is higher in human eggs inseminated by partial zona dissection at later times after egg retrieval, suggesting that aged human eggs may be less able to establish a membrane block to polyspermy (Malter et al., 1989).

Unlike non-mammalian species, the mechanism of the mammalian membrane block does not appear to be a change in egg membrane potential as significant membrane depolarization has not been observed in fertilized mammalian eggs (Miyazaki and Igusa, 1981; Igusa et al., 1983; Jaffe et al., 1983; McCulloh et al., 1983; Jaffe et al., 1985). We have recently shown that perturbation of the egg actin cytoskeleton or of sperm-induced calcium signalling leads to increased polyspermy during IVF of zona pellucida-free mouse eggs, suggesting that egg actin and calcium signalling are involved in the establishment of a membrane block to polyspermy (McAvey et al., 2002). As aged eggs have been reported to have abnormalities in both sperm-induced calcium signalling (Jones and Whittingham, 1996; Igarashi et al., 1997; Gordo et al., 2002) and the actin cytoskeleton (Webb et al., 1986; Pickering et al., 1988), we test the hypothesis that aged eggs have a reduced ability to establish a membrane block to prevent polyspermy. This also addresses the issue of increased polyspermy with post-ovulatory ageing; while some studies detect an increased incidence of polyspermy in vivo with increased time after ovulation (Austin and Braden, 1953a,b; Odor and Blandau, 1956; Yanagimachi and Chang, 1961), other studies do not report such an observation (Braden and Austin, 1954; Gates and Beatty, 1954; Chang and Fernandez-Can, 1958; Adams and Chang, 1962; Marston and Chang, 1964). These different observations from in vivo studies may be due in part to methodological factors, including different methods for insemination (natural mating or artificial insemination), assessment of polyspermy at different developmental stages (a few hours to 3.5 days after insemination or ovulation), differences in the methods for identifying sperm penetration and the difficulties in distinguishing polyspermy from pollygyn (the latter resulting from failed meiotic progression). Our work here advances these previous studies of post-ovulatory ageing by examining sperm–egg membrane interactions and the establishment of the membrane block to polyspermy.

Materials and methods

Egg collection, zona pellucida removal and sperm preparation

Egg collection and zona pellucida removal were performed as previously described (McAvey et al., 2002). Metaphase II-arrested eggs were collected from 6 to 8-week-old ovulated inactivated CF-1 mice (Harlan, Indianapolis, IN) at either 13 h (referred to as young eggs) or 22 h (aged eggs) post-HCG injection to induce ovulation. Mice ovulate at ~10–13 h post-HCG injection (Marston and Chang, 1964; Hogan et al., 1986). These post-HCG times were chosen based on previous studies of post-ovulatory ageing on mouse eggs (Xu et al., 1997; Abbott et al., 1998).

Cumulus cells were removed by brief incubation (<5 min) in either Whitten’s medium [109.5 mM NaCl, 4.7 mM KCl, 1.2 mM KH2PO4, 1.2 mM MgSO4, 5.5 mM glucose, 0.23 mM pyruvic acid, 4.8 mM lactic acid (Whitten, 1971)] with 7 mM NaHCO3 and 15 mM HEPES (hereafter referred to as ‘Whitten’s-HEPES’) and 0.04% Type I-S hyaluronidase (Sigma, St Louis, MO), or in Whitten’s-HEPES medium containing 30 mg/ml bovine serum albumin (BSA) (Albumax I from Gibco-BRL, Gaithersburg, MD) and 0.02% Type IV-S hyaluronidase (Sigma). After cumulus cell removal, the zona pellucida were removed by a brief incubation (~15 s) in acidic culture medium compatible buffer (10 mM HEPES, 1 mM NaH2PO4, 0.8 mM MgSO4, 5.4 mM KCl, 116.4 mM NaCl, final pH 1.5) and then allowed to recover for 60 min in Whitten’s medium containing 22 mM NaHCO3 and 15 mg/ml BSA. Eggs were cultured in 5% CO2 in air.

Sperm were collected from the cauda epididymides of sacrificed CD1 male mice (8-week-old or retired breeders, Harlan, Indianapolis, IN). Sperm from one epididymis were collected in 100 μl Whitten’s medium containing 15 mg/ml BSA. After 10–15 min, the tissue was removed from the droplet and the sperm were pipetted into the bottom of a tube containing 750 μl Whitten’s medium. After 45 min, 225 μl from the top of the swim-up culture was removed and placed in a culture dish and covered with light mineral oil. The sperm were cultured for a total of 2.5–3 h in Whitten’s medium containing 15 mg/ml BSA to allow the sperm to undergo capacitation and spontaneous acrosome exocytosis. Sperm concentration was determined using a hemocytometer and sperm/ml was determined.

IVF

Zona pellucida - free eggs were inseminated in 10 μl culture medium drops, with 10 eggs per 10 μl insemination drop, with 100,000 sperm/ml unless otherwise indicated. These insemination conditions resulted in nearly 100% fertilization of molecules and the sperm were pipetted into the bottom of a tube containing 750 μl Whitten’s medium. After 45 min, 225 μl from the top of the swim-up culture was removed and placed in a culture dish and covered with light mineral oil. The sperm were cultured for a total of 2.5–3 h in Whitten’s medium containing 15 mg/ml BSA to allow the sperm to undergo capacitation and spontaneous acrosome exocytosis. Sperm concentration was determined using a hemocytometer and sperm/ml was determined.

In vitro re-insemination experiments

Figure 2 shows an IVF assay, called a re-insemination experiment, used for assessment of the membrane block to polyspermy (Wolf, 1978; Horvath et al., 1993; Sengoku et al., 1995). The goal of the re-insemination experiment was to assess the ability of fertilized zona pellucida-free eggs to be fertilized by additional sperm, which is indicative of an inadequate membrane block to polyspermy. Initial control experiments for these studies compared untreated eggs to eggs treated with 10 μM 1,2-bis(o-aminophenyl)ethane-N,N,N′,N′-tetra-acetic acid acetoxyethyl ester (BAPTA-AM; Calbiochem, La Jolla, CA), which we have previously shown leads to increased polyspermy in conventional IVF assays (McAvey et al., 2002). Eggs were incubated in 10 μM BAPTA-AM [diluted in Whitten’s medium from a 100 mM stock in dimethyl
sulphoxide (DMSO) for 1 h, and then washed prior to insemination as previously described (McAvey et al., 2002). Control eggs were incubated in 1% DMSO, which we have previously shown does not affect sperm penetration (Evans et al., 2000; McAvey et al., 2002).

The first insemination in these re-insemination experiments (IVF1) was optimized to generate monospermic eggs (data not shown). The reason for this was polyspermic eggs show a higher frequency of calcium oscillations, and these altered calcium oscillations could possibly affect egg activation events, including the membrane block to polyspermy (Faure et al., 1993). The conditions for the first insemination (IVF1) that produced primarily monospermic eggs for each of the experimental groups were as follows: control eggs, 50000 sperm/ml for 45 min; aged eggs, 100000 sperm/ml for 45 min; BAPTA-AM-treated eggs, 30000 sperm/ml for 45 min. After this first insemination, eggs were washed through one 100 µl drop of Whitten’s medium containing 0.1% polyvinyl alcohol to remove loosely attached sperm, and then three 100 µl drops of Whitten’s medium containing 15 mg/ml BSA. A subset of eggs (10–20 eggs per experimental group) were fixed and mounted so that the extent of fertilization after the first insemination could be assessed. The remainder of the fertilized eggs were incubated for 3 h in a sperm-free drop, since ~1–2 h appears sufficient to allow the establishment of the membrane block to polyspermy (Wolf, 1978; Horvath et al., 1993; Sengoku et al., 1995; Redkar and Olds-Clarke, 1999; McAvey et al., 2002). After this 3 h incubation, eggs that had emitted a second polar body were selected for a second insemination (‘re-insemination’). Eggs with second polar bodies were selected in order to omit eggs that failed to fertilize in the first insemination. However, because aged eggs are more susceptible to parthenogenetic activation, it was possible that aged eggs with second polar bodies included spontaneously activated eggs (determined by the absence of a male pronucleus); this was taken into account in our data analyses. Furthermore, BAPTA-AM-treated eggs do not emit second polar bodies, and therefore all BAPTA-AM-treated eggs were used in the second insemination. The second insemination used 100 000 sperm/ml for 1.5 h; sperm for this second insemination were freshly collected and capacitated for 2–3 h. As a control, unfertilized eggs from each experimental group were inseminated in parallel with the fertilized egg groups to assess the baseline level of the average number of sperm fused per egg from this second insemination. At the end of the second insemination, eggs were washed, fixed and stained with DAPI, and then were assessed for the average number of sperm fused per egg, and whether these were decondensed sperm heads (indicative of having fertilized the eggs recently, from the second insemination) or were forming male pronuclei (indicative of the sperm coming from the first insemination). Data from these experiments were analysed using analysis of variance with Fisher’s PLSD post hoc testing or χ² analysis using StatView version 5.0 (SAS Institute); P < 0.05 was considered significant.

Egg CG staining and quantification

Eggs were fixed in 3.7–4.0% freshly prepared paraformaldehyde in PBS, permeabilized in PBS containing 0.1% Triton-X 100 for 15 min, and then incubated in Lens culinaris agglutinin (LCA) block (PBS containing 3 mg/ml NaIO₄-treated casein, 0.1 M glycine). CGs were labelled with 10 µg/ml LCA-biotin followed by 2–3 µg/ml fluorescent avidin (Vector Laboratories). Eggs were then mounted on 4 µl Vectashield mounting medium (Vector Laboratories) to flatten them between the slide and coverslip; the mounting medium also contained 1.5 µg/ml DAPI to stain DNA. For each egg, the CGs in a small area (50 µm²) were counted by hand and by computer-assisted image quantification, and a large area (300 µm²) was counted by computer-assisted image quantification (Abbott et al., 1999). These values were compared to verify that they agreed, and the data for each egg were expressed as number of CGs per 100 µm².

Egg actin and β-tubulin staining

Eggs were fixed in 3.7–4.0% freshly prepared paraformaldehyde in PBS, permeabilized in PBS containing 0.1% Triton-X 100 for 15 min, and then incubated in PBS containing 0.1% BSA and 0.01% Tween 20 (IIF block) for 45 min to block non-specific binding sites. Actin was stained with Alexa 546-conjugated phallolidin (5 units/ml; Molecular Probes; Eugene, OR) and β-tubulin was labelled with an anti-β-tubulin antibody (1:100 dilution of mouse monoclonal ascites fluid; Zymed, South San Francisco, CA) followed by a goat anti-mouse immunoglobulin G fluorescein isothiocyanate-conjugated secondary antibody (1:250 dilution; Jackson Immunoresearch; West Grove, PA). The eggs were washed 3–6 times in IIF block after each antibody incubation and then mounted in Vectashield mounting medium containing 1.5 µg/ml DAPI. All images for each fluorophore were photographed using the same exposure time.

Results

IVF experiment no. 1: incorporation of sperm over time

Through a series of IVF assays, we examined the ability of post-ovulatory aged eggs to support sperm–egg membrane interactions

Figure 1. Effect of post-ovulatory ageing on sperm–egg fusion and the extent of fertilization. Zona pellucida-free young and aged eggs (13 and 22 h post-HCG, respectively) were inseminated for 0.75, 1.5, 2.5, or 4 h with 100 000 sperm/ml, then washed to remove any loosely attached sperm, fixed and stained with DAPI to assess sperm–egg fusion. Results are based on three experiments and 50–120 total eggs for each time point. Panel A shows the average number of sperm fused per egg. Statistically significant differences between aged eggs (open circles) and the young egg time-matched controls (closed squares) are indicated with asterisks (P < 0.05). Panel B shows the percentage of eggs fertilized for each group at each time point. These data are shown in more detail with χ² statistical analysis in Table I.
and the ability of aged eggs to establish a membrane block to prevent polyspermy. These IVF assays used zona pellucida-free mouse eggs collected from oviducts at 13 and 22 h post-HCG (hereafter referred to as ‘young’ and ‘aged’, respectively)—post-HCG time points that have been used for previous studies of post-ovulatory ageing of mouse eggs (Xu et al., 1997; Abbott et al., 1998). The first series of IVF assays examined the extent of sperm–egg fusion over time, measured as the average number of sperm fused per egg. The number of sperm fused per zona pellucida-free egg plateaus with increased time post-insemination, indicative of the establishment of a membrane block to polyspermy that prevents additional sperm from fusing with fertilized eggs (Wulf, 1978; Sengoku et al., 1995; Redkar and Olds-Clarke, 1999; McAvey et al., 2002). Young and aged zona pellucida-free eggs were inseminated with 100 000 sperm/ml, and then sperm–egg fusion and the percentage of eggs that fertilized were assessed at 0.75, 1.5, 2.5 and 4 h post-insemination (Figure 1).

Aged eggs were slower to fertilize than young eggs, as indicated by a lower average number of sperm fused per egg (Figure 1A) and a lower percentage of eggs fertilized (Figure 1B) at 0.75 and 1.5 h post-insemination. At 2.5 and 4 h post-insemination, the average number of sperm fused per egg and the percentage of eggs fertilized were similar in young and aged eggs. Statistical analysis ($\chi^2$-test) of the distribution of the numbers of sperm fused per egg revealed that there was a statistically significant difference between young and aged eggs at 0.75, 1.5 and 4 h post-insemination (Table I).

### Table I. Comparisons of sperm distributions of young and aged eggs at 0.75, 1.5 or 4 h post-insemination (100 000 sperm/ml)

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<th>No. of sperm fused per egg</th>
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<tr>
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<td>No. of sperm fused per egg</td>
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<td>Young eggs (n = 53)</td>
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<td>3.0% (1/33)</td>
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<td>1</td>
<td>78.0% (26/33)</td>
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<tr>
<td>2</td>
<td>12.1% (4/33)</td>
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**IVF experiment no. 2: challenging fertilized eggs with additional sperm**

The delay in fertilization of aged eggs made it difficult to use sperm incorporation over time as a means to assess establishment of a membrane block to polyspermy, as has been done previously (Wulf, 1978; Sengoku et al., 1995; Redkar and Olds-Clarke, 1999; McAvey et al., 2002). To evaluate the ability of aged eggs to establish a membrane block to polyspermy, we performed re-insemination experiments in which zygotes (recently fertilized eggs) were challenged with fresh sperm in a second insemination (‘re-insemination’) (Figure 2). These re-insemination experiments have three different samples. The ‘IVF1+2’ eggs were first fertilized with one batch of sperm to generate fertilized, monospermic zygotes; these zygotes were then challenged in a re-insemination with a second batch of sperm. The two controls are ‘IVF1’, eggs that were only inseminated with the first IVF, and ‘IVF2’, eggs that were only inseminated with the second IVF. To verify experimental conditions for these re-insemination studies, we first compared BAPTA-AM-treated eggs to control (untreated) eggs, as eggs treated with 10 $\mu$M BAPTA-AM experience no fertilization-induced increases in $[\text{Ca}^{2+}]_{\text{cyt}}$ (Kline and Kline, 1992 and our unpublished data) and become significantly more polyspermic than control zona pellucida-free eggs in studies of sperm incorporation over time (McAvey et al., 2002). We, therefore, predicted that fertilized BAPTA-AM-treated eggs would incorporate sperm from a second insemination whereas control eggs would not. Indeed, the average number of...
sperm fused per BAPTA-AM-treated egg was statistically significantly higher for IVF1 + 2 (4.76 ± 0.22 sperm fused/egg) as compared to IVF1 (2.12 ± 0.20 sperm fused/egg) (Figure 3); this demonstrates that fertilized BAPTA-AM-treated eggs continue to incorporate sperm from the second insemination, indicative of a reduced ability to establish a membrane block to polyspermy (Wolf, 1978; Zuccotti et al., 1991; Horvath et al., 1993; Maluchnik and Borsuk, 1994). In contrast, the average number of sperm fused per control (untreated) eggs were similar for IVF1 + 2 and IVF1 (1.51 ± 0.11 sperm fused/egg and 1.30 ± 0.11 sperm fused/egg, respectively), and only three of the 62 control eggs were fused with sperm from the second insemination. These data demonstrate that control eggs establish a membrane block to polyspermy, that BAPTA-AM-treated eggs have a reduced ability to establish a membrane block in agreement with our previous study (McAvey et al., 2002) and also confirm the validity of our re-insemination experimental design.

We next used the re-insemination IVF assay to test the hypothesis that aged eggs have a reduced ability to establish a membrane block to polyspermy. As shown in Figure 2 (inset), sperm from the first insemination were observed in the early stages of forming a male pronucleus (Figure 2 inset, MPN) whereas sperm from the second insemination were observed as decondensing sperm heads in the egg cytoplasm (Figure 2 inset, DS). Thus, sperm from the first insemination could be distinguished from sperm from the second insemination. These experiments revealed that fertilized aged eggs were penetrated by significantly more sperm from the second insemination than fertilized young eggs. As shown in the IVF1 + 2 values in Figure 4, aged eggs had 0.56 ± 0.10 decondensing sperm per egg, whereas young eggs had 0.02 ± 0.02 decondensing sperm per egg. Only one decondensing sperm was found in 55 young eggs examined (Table II), indicating that virtually no sperm from the second insemination were able to penetrate fertilized young eggs. In contrast, 27 of 113 fertilized aged eggs (24%) were penetrated by decondensing sperm from the second insemination (Table II). These data clearly show that zygotes from aged eggs could still be fertilized by additional sperm, indicating that aged eggs have an impaired ability to establish a membrane block to polyspermy.

Results from these re-insemination experiments also demonstrate the reduced fertilizability of aged eggs. Nearly 20% of the aged eggs (28/141) were not fertilized after the two inseminations (Table II). In contrast, 100% of the young eggs were fertilized in the first insemination. Together with the data in Figure 1, these data provide further evidence for the reduced ability of the aged egg membrane to support sperm–egg membrane interactions.

Figure 4. Effect of post-ovulatory ageing on the formation of the membrane block to polyspermy. Zona pellucida-free young and aged eggs were inseminated for 45 min with 100 000 sperm/ml (aged) or 50 000 sperm/ml (young), after which eggs were washed to remove loosely attached sperm. A subset of eggs from both groups was fixed at this point (IVF1). Fertilized eggs were incubated for 3 h in sperm-free drops, then re-inseminated for 1.5 h with 100 000 sperm/ml (IVF1+2), in parallel with unfertilized young and aged eggs (IVF2). The average numbers of sperm fused per egg for the IVF2 controls were 3.00 ± 0.22 for young eggs and 2.90 ± 0.26 for aged eggs. Results are based on three experiments and 50–140 eggs for each group, with the graph showing the average number of sperm fused per fertilized egg for each insemination. Sperm were characterized as those from the first insemination (solid bars, pronuclear stage in IVF1+2) or second insemination (hatched bars, decondensing sperm heads; see also the inset in Figure 2). Statistically significant differences in the number of decondensed sperm in re-inseminated eggs are indicated with an asterisk (*P < 0.0001).

Figure 3. Effect of BAPTA-AM on the establishment of the membrane block to polyspermy. Zona pellucida-free eggs (collected at 13 h post-HCG) were incubated in medium containing 10 µM BAPTA-AM or the vehicle control, 1% DMSO, for 1 h prior to insemination. Eggs were then inseminated for 45 min with 30 000 (BAPTA-AM) or 50 000 sperm/ml (control), after which the eggs were washed to remove loosely attached sperm. Subsets of control and BAPTA-AM-treated eggs were fixed at this point (IVF1). Remaining eggs were incubated for 3 h in sperm-free drops and then re-inseminated for 1.5 h with 100 000 sperm/ml (IVF1+2), in parallel with unfertilized BAPTA-AM-treated and control eggs (IVF2). The average numbers of sperm fused per egg for the IVF2 controls were 2.55 ± 0.22 for control eggs and 4.26 ± 0.22 for BAPTA-AM-treated eggs. Results are based on three experiments and 40–60 total eggs for each group. The graph shows the average number of sperm fused per fertilized egg for each insemination. (Fourteen control eggs and ten BAPTA-AM-treated eggs were omitted from this analysis because they were not fertilized in IVF1.) IVF1 and IVF1 + 2 values for control eggs are not statistically different, while the difference observed between IVF1 and IVF1 + 2 for BAPTA-AM-treated eggs is statistically significant (*P < 0.0001; asterisk).
Aged eggs had 1.48 ± 0.3 sperm bound per egg. After 15 min inseminations with 100 000 sperm/ml, young eggs had 6.27 ± 0.3 sperm bound per egg, and aged eggs had 7.07 ± 0.32 sperm bound per egg (P > 0.05). After 60 min inseminations with 100 000 sperm/ml, young eggs had 2.13 ± 0.36 sperm bound per egg, and aged eggs had 1.48 ± 0.26 sperm bound per egg (P > 0.05).

### Fertilization-induced CG exocytosis

The reduced ability of aged eggs to establish a membrane block to polyspermy could be due to an overall reduced ability to mount egg activation responses following fertilization. To assess aged egg responsiveness to sperm, we investigated another egg activation event and post-fertilization membrane dynamics, CG exocytosis, by examining CG density in young and aged eggs after fertilization (Figure 5). In agreement with previous studies (Xu et al., 1997), we observed that CG density in unfertilized aged eggs was reduced by 40% as compared to unfertilized young eggs (Figure 5; P < 0.0001). As a result of this pre-fertilization CG release occurring with post-ovulatory ageing, aged eggs had fewer CGs at the start of the insemination and released 42% of these CGs by 4 h post-insemination. Young eggs, starting with more CGs before fertilization, released 70% of their CGs by 4 h post-insemination. As a result of sperm-induced exocytosis, young and aged eggs had similar CG densities at 1.5 and 4 h post-insemination (P > 0.05). The residual CG staining observed at these post-insemination time points may be CGs that have fused with the egg plasma membrane, but have not yet completely released their contents (Abbott and Ducibella, 2001), or may be CGs that have not yet been exocytosed.

### Cytoskeletal organization

Since we have previously shown that perturbation of the egg actin cytoskeleton can lead to increased polyspermy or decreased fertilization (McAvey et al., 2002), we examined if cytoskeletal abnormalities were present at 22 h post-HCG (~10 h post-ovulation), possibly contributing to the reduced fertilizability and impaired membrane block establishment. Young metaphase II eggs have an actin-rich cap overlying the meiotic spindle (Maro et al., 1984) (Figure 6A and D). Aged eggs collected at 22 h post-HCG displayed two abnormal actin distributions. Some aged eggs (5–25% of the eggs collected on a given day) had no obvious actin-rich cap over the meiotic spindle, similar to what was observed in mouse eggs collected 24–48 h post-HCG (Webb et al., 1986) (Figure 6C). Other aged eggs (50–75% of the eggs collected on a given day) had a dramatic actin-rich protrusion over the meiotic spindle (Figure 6B). These eggs were not undergoing spontaneous exit from metaphase II, as indicated by the presence of an intact meiotic spindle under this actin-rich protrusion (Figure 6E and F). We also stained eggs for β-tubulin to visualize microtubules. In young eggs, β-tubulin was localized to the meiotic spindle (Figure 6G and J). In aged eggs, β-tubulin was localized to the meiotic spindle and microtubule aster-like bundles in the egg cytoplasm and cortex (Figure 6H and I). These aster-like bundles have previously been observed in much older mouse and human eggs (~15–28 h after ovulation (Eichenlaub-Ritter et al., 1986) or ~61–87 h after HCG (Pickering et al., 1988)).

### Sperm–egg adhesion

Since sperm binding to the egg membrane is a prerequisite for gamete membrane fusion, we hypothesized that the decreased fertilizability of aged eggs (Figures 1 and 4; Table II) was due to reduced sperm binding to the egg membrane. However, two IVF assays to assess sperm–egg binding revealed no difference in the number of sperm bound per egg between aged and young eggs. After 15 min inseminations with 100 000 sperm/ml, young eggs had 6.27 ± 0.3 sperm bound per egg, and aged eggs had 7.07 ± 0.32 sperm bound per egg (P > 0.05). After 60 min inseminations with 100 000 sperm/ml, young eggs had 2.13 ± 0.36 sperm bound per egg, and aged eggs had 1.48 ± 0.26 sperm bound per egg (P > 0.05).

### Figure 5. Effect of post-ovulatory ageing on the extent of CG exocytosis after insemination.

Zona pellucida-free young and aged eggs were inseminated for 1.5 or 4 h with 100 000 sperm/ml, then fixed and stained with fluorescently conjugated LCA to label CGs. The graph shows the average number of CGs per 100 μm² in unfertilized eggs (time 0 h) and fertilized eggs (at 1.5 and 4 h post-insemination). Closed squares represent young eggs, and open circles represent aged eggs. Results are based on three experiments and 20–30 total eggs for each time point. Unfertilized aged eggs had a significantly lower density of CGs compared to unfertilized young eggs (P < 0.0001). The CG densities in fertilized young and aged eggs were not statistically different at 1.5 or 4 h post-insemination (P > 0.05).

### Discussion

This study demonstrates that one deficiency in aged eggs is the reduced ability to mount a membrane block to polyspermy (Figure 4, Table II). Although the mechanism of the membrane block to polyspermy in mammalian eggs is largely unknown, we have recently shown that the membrane block to polyspermy, requires increases in intracellular calcium that occur upon fertilization and that zona pellucida-free eggs treated with the actin-disrupting drug cytochalasin D become more polyspermy than controls when inseminated (McAvey et al., 2002). We have also observed that attenuation of sperm-induced Ca²⁺ signalling can affect the quality of the membrane block (Gardner and Evans, unpublished data). In light of this, the altered sperm-induced calcium signalling observed in aged eggs (Jones and Whittingham, 1996; Igarashi et al., 1997;
Gordo et al., 2002) is a possible factor contributing to the reduced ability to establish the membrane block. In addition, we observed abnormalities in actin organization in eggs collected at 22 h post-HCG (Figure 6), demonstrating that cytoskeletal alterations identified in previous studies of mouse and human eggs, 24–87 h post-HCG induction or, 15 h after ovulation (Eichenlaub-Ritter et al., 1986; Webb et al., 1986; Pickering et al., 1988) are present at earlier times during post-ovulatory ageing. This abnormal actin organization in aged eggs could affect the establishment of the membrane block to polyspermy. The abnormal actin organization could adversely affect the ability of the egg to undergo sperm-induced membrane rearrangements that may result in decreased post-fertilization receptivity to sperm. Actin may also affect Ca$^{2+}$ influx (Rosado and Sage, 2000), raising the possibility that the abnormal actin organization in aged eggs may alter sperm-induced Ca$^{2+}$ signalling which would in turn disrupt membrane block establishment. It should be noted that the reduced ability of aged eggs to establish a membrane block to polyspermy is not due to a complete inability to respond to sperm and mount egg activation responses, as we find that fertilization induces CG exocytosis in young and aged eggs (Figure 5). These data also suggest that aged eggs can establish at least a partial zona pellucida block to polyspermy. It is not clear, however, if CG exocytosis and/or an exocytosis-associated event plays a role in the membrane block to polyspermy.

Post-ovulatory ageing also appears to have deleterious effects on the ability of the aged egg membrane to support interactions with sperm. Our assays of sperm–egg binding demonstrate similar levels of sperm binding to young and aged zona pellucida-free eggs, suggesting that aged eggs have a reduced ability to support sperm fusion or possibly to establish firm pre-fusion adhesions. We have conducted studies on an egg membrane protein, CD9, that is known to be essential for gamete membrane interactions (Kaji et al., 2000; Le Naour et al., 2000; Miyado et al., 2000), but we have thus far not found any obvious differences in the amount or localization of CD9 on young and aged eggs (data not shown), although it is possible that ageing induces more subtle alterations in CD9 or in other egg molecules. The abnormalities in the actin cytoskeleton in aged eggs could contribute to the reduced receptivity to sperm. Egg surface topography (e.g. arrangement of microvilli on the surface) and cortical cytoskeletal networks appear to be important for sperm–egg

Figure 6. Fluorescent micrographs of young and aged eggs stained for actin, β-tubulin and chromatin. Zona pellucida-free young and aged eggs (13 and 22 h post-HCG, respectively) labelled with phalloidin to stain F-actin (panels A, B, C) or a monoclonal antibody to β-tubulin (panels G, H, I). Panel I is an adjusted version of Panel H, with the greyscale manipulated in Photoshop to show more contrast between the fluorescence of the asters and the background. Panels D, E, F and J, K, L show corresponding DAPI staining to the panels directly above. A mouse egg is ~80 μm in diameter. Young eggs: panels A, D, G, J; Aged eggs: panels B, C, E, F, H, I, K, L.
membrane interactions (Phillips and Shalgi, 1982; Talbot and Chacon, 1982; Phillips et al., 1985; Webster and McGaughy, 1990), and these are very likely to be affected by actin microfilament organization in the egg. The actin cytoskeleton in the egg may also mediate clustering of egg binding sites for sperm ligands that mediate sperm–egg fusion; such clustering of binding sites for increased avidity is known to be important for other cellular interactions (van Kooyk and Figdor, 2000). Additionally, it is possible that the preocious exocytosis of CGs during post-ovulatory ageing (Xu et al., 1997; Figure 5) alters the egg membrane so that sperm–egg membrane interactions are not favoured.

These data along with complementary studies provide insights into how post-ovulatory ageing contributes to reproductive failures. In our experiments with zona pellucida-free mouse eggs, polyspermic fertilization (failed membrane block) and no fertilization (failed gamete membrane interactions) occurred with similar frequencies. Other studies on fertilization (in vivo, in vitro and ICSI) of aged eggs also provide evidence of decreased fertilization and increased polyspermy, suggesting that both these factors contribute to reproductive failures with aged eggs (Ben-Rafael et al., 1986; Badenass et al., 1989; Malter et al., 1989; Pool et al., 1990; Winston et al., 1993; Goud et al., 1999; Park et al., 2000; Kuczynski et al., 2002).

An analysis of pregnancies in women who likely conceived at later times after ovulation indicates that these women experience significantly higher rates of early pregnancy loss than women who conceive at times closer to the time of ovulation (Wilcox et al., 1998). Polyspermic embryos and also other factors affecting embryo quality could be the underlying causes of this early pregnancy loss. The reduced ability of aged eggs to establish a membrane block also raises the possibility that they undergo suboptimal egg activation in response to sperm, and thus aged eggs may have deficiencies in the transition from egg to embryo. This is supported by the observation that aged eggs have lower rates of successful fertilization by ICSI and of subsequent embryo development (Goud et al., 1999), and by data demonstrating that rescue insemination by ICSI is more successful (i.e. higher fertilization and implantation rates) if performed soon after the first insemination attempt (Yuzpe et al., 2000; Chen and Kattera, 2003).

This study provides new insights into egg membrane function in aged eggs, complementing studies that document other changes associated with post-ovulatory ageing. Further characterization of the aged egg membrane function awaits advances in our understanding of the molecular basis of sperm–egg interactions and egg membrane dynamics following fertilization. Examination of plasma membrane dynamics after fertilization in young and aged eggs may also provide insights into the mechanisms underlying the membrane block to polyspermy.

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Egg ageing and the membrane block to polyspermy


