Sperm-induced oocyte activation in the rhesus monkey: nuclear and cytoplasmic changes following intracytoplasmic sperm injection

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Intracytoplasmic sperm injection (ICSI) has increased the potential of the assisted reproductive technologies to propagate mammalian species and has provided an opportunity for research into cell cycle control and the mechanisms involved in sperm-induced oocyte activation. We have investigated the efficacy of ICSI in the rhesus monkey, the mechanism of fertilization following sperm injection and the cytoskeletal rearrangement that occurs upon oocyte activation. These studies were conducted on mature, and to a lesser extent, immature oocytes. Ejaculated spermatozoa, washed, capacitated and activated before immobilization, were injected into oocytes using conventional ICSI methodology. Sperm injection into mature oocytes induced oocyte activation (19/22; 86%) and pronuclear formation. In contrast, sham-injected oocytes did not activate readily (2/16; 13%). To localize oocyte activation factor(s), spermatozoa were separated mechanically into heads and tails which were then injected individually into mature oocytes. Activation occurred in 87% (20/23) of oocytes receiving heads. After tail injection, a single microtubule asters was nucleated and one pronucleus (PN) was seen in four of 21 oocytes. Intracytoplasmic injection of sperm extract (SE) resulted in oocyte activation at a significantly higher rate than occurred following sham injection (76 versus 13%). Sperm-induced oocyte activation was also evaluated in immature metaphase (MI) oocytes; activation occurred in 46% (12/26) of cases; however, only 8% of the activated oocytes exhibited 2 PN. Finally, β-tubulin localization in untreated and taxol-treated oocytes was established as a marker for cytoplasmic changes associated with oocyte activation. These results are consistent with the hypothesis that spermatozoa contain an oocyte activating factor(s) which is primarily localized in the sperm head. Moreover, an activation response is limited to mature oocytes and is accompanied by cytoskeletal changes analogous to those seen following conventional fertilization.

Key words: intracytoplasmic sperm injection/microtubules/oocyte activating factor/oocyte activation

Introduction

Success with intracytoplasmic sperm injection (ICSI) has been reported in rabbits (Hosoi et al., 1988) and cattle (Goto et al., 1990) and, more recently, in the mouse (Kimura and Yanagimachi, 1995). Clinically, this procedure has been widely employed as a treatment for male factor infertility, using ejaculated, epididymal or testicular spermatozoa (Palermo et al., 1992; Van Steirteghem et al., 1993, 1996; and Palermo et al., 1996). However, the mechanism of oocyte activation during ICSI is still a matter for discussion (Edwards and Van Steirteghem, 1993). During the fertilization process, only capacitated spermatozoa are capable of binding to, fusing with, and penetrating the oolemma. This fusion of the male and female gametes initiates a cascade of events in the oocyte, termed activation, evidence for which includes the cortical reaction, resumption of meiosis (transition from meiotic metaphase to mitotic interphase), increased metabolic activity and cytoskeletal remodelling (Albertini 1987, 1992; Yanagimachi, 1994; Van Blerkom et al., 1995). The process of ICSI obviously bypasses the sperm–oocyte membrane fusion step in fertilization. Three possible mechanisms for oocyte activation secondary to the ICSI procedure include: transient oocyte exposure to high extracellular calcium; mechanically induced parthenogenesis (Perreault and Zirkin, 1982; Lanzendorf et al., 1988; Edwards and Van Steirteghem, 1993); and the release of a sperm-associated factor into the ooplasm (Edwards and Van Steirteghem, 1993; Tesarik et al., 1994). Recently Parrington et al. (1996) identified a soluble sperm protein from hamster sperm that induces Ca$^{2+}$ oscillations in eggs. This factor, termed oscillin, may represent the physiological trigger for development in mammals.

Since the reproductive physiology of the rhesus monkey is similar to that of the human, experimental studies on the biology of fertilization in this species may lead to clinical application. Moreover, the development of ICSI in the non-human primate carries implications for the preservation of endangered species where sperm capacitation is limited or when only submotile spermatozoa may be available. The purpose of this study was to establish the efficacy of ICSI in the monkey and to explore the mechanism of oocyte activation following ICSI. We were also interested in establishing cytoplasmic markers to evaluate oocyte activation which could be used in conjunction with nuclear analysis to monitor the activity of a sperm-associated, oocyte activation factor. With this aim, the localization of β-tubulin was examined in untreated and taxol-treated oocytes, to assess its use as a marker for cytoplasmic changes associated with activation.
Materials and methods

Oocyte recovery and preparation

Rhesus monkeys were pretreated for 7 days with a gonadotrophin-releasing hormone (GnRH) antagonist (Antide; Laboratoires Serono SA, Aubonne, Switzerland; 1.0 mg/kg body weight, s.c., once daily) which continued during administration of either recombinant human follicle-stimulating hormone (r-hFSH) alone (r-hFSH; Ares Serono; 30 IU, i.m., twice daily) or sequential treatment with r-hFSH (for 6 days) followed by r-hFSH + recombinant human luteinizing hormone (r-LH) (Ares Serono; 30 IU each, i.m., twice daily for 1–3 days). When ultrasonography revealed follicles ~4 mm diameter, human chorionic gonadotrophin (HCG) (Serono; 1000 IU, i.m.) was given the next day. Follicles were aspirated by laparotomy or laparoscopy, 27 h post-HCG. The pooled aspirates from left and right ovaries were immediately transferred to the laboratory for oocyte recovery. Oocytes were evaluated for maturity and transferred to equilibrated culture medium (TALP) with 0.3% BSA overlaid with heavy white mineral oil saturated with TALP.

Sperm extract (SE) preparation

Rhesus monkey semen was collected by penile electroejaculation from proven fertile donors. The ejaculate was washed twice in TALP-HEPES (pH 7.4), and once in Dulbecco's modified phosphate-buffered saline (DPBS) before the sperm concentration was determined using a haemocytometer. The final suspension (1×10⁶ spermatozoa/ml) was lysed by one freeze–thaw cycle in liquid nitrogen, held for 1 h at room temperature, and centrifuged at 1300 g at room temperature for 10 min to remove particulates. The supernatant was further centrifuged at 100 000 g at 4°C, divided into aliquots of 50 μl and stored in liquid nitrogen.

In-vitro fertilization (IVF)

Ejaculates were diluted 1:30 with TALP-HEPES (pH 7.4) and washed twice (360 g, 7 min). After washing, final pellets were resuspended in TALP containing 0.3% BSA and incubated in 5% CO₂: 95% air at 37°C for 4 h. Spermatozoa were activated by No. 053H4806; Sigma; 1:200, 40 min at 37 °C in 100% humidity). Ejaculates were diluted 1:30 with TALP-HEPES (pH 7.4) and subsequently immobilized by gently stroking its mid-piece with an oil-saturated 10% polyvinyl pyrrolidone (PVP; MW 360 000) under mineral oil. A motile spermatozoon was selected for injection from the latter drop and subsequently immobilized by gently stroking its mid-piece with the injection pipette.

To separate sperm heads and tails, an immobilized spermatozoon was allowed to attach to the bottom of a Petri dish. The head was gently aspirated into an injection pipette which was moved suddenly toward the holding pipette, decapitating the sperm. Individual heads or tails were then isolated for subsequent microinjection. For sperm extract and sham injections, oocytes were injected with either ~5 pl of DPBS containing extract harvested from two to five spermatozoa or the same volume of DPBS alone. This injection volume was calculated from the inner diameter of the pipette and the estimated length of the injected column. The injection procedure was performed as described by Dozortsev et al. (1994) and Rybouchkin et al. (1995). Fifteen hours after microinjection or after conventional insemination with spermatozoa (2×10⁵ motile cells/ml) at 37°C, oocytes were examined using Hoffman optics for the presence of pronuclei.

Taxol treatment

Taxol (Sigma) was dissolved in dimethyl sulfoxide (DMSO) at a stock concentration of 1 mM and stored in the dark at −20°C. The stock solution was thawed just prior to dilution to KSOM (Lawitts and Biggers, 1993) + 10% NCS. At the end of each culture period, experimental and control oocytes were treated for 15 min in KSOM + 10% NCS + 1% DMSO with or without 10 mM taxol prior to processing for fluorescence microscopy.

Oocyte fixation and permeabilization

Zona pellucidae were removed following a brief exposure to acidified TALP-HEPES + 0.3% BSA (pH 2.5). After three washes in TALP-HEPES + 0.3% BSA and a 30 min recovery, zona-free oocytes were equilibrated for 1 min in Ca²⁺, Mg²⁺-free PBS in an agarose (5%) coated culture dish. Each oocyte was then carefully transferred onto a poly-l-lysine-covered coverslip overlaid with Ca²⁺, Mg²⁺-free PBS. Following a 2–3 min attachment period at 37°C, oocytes were fixed in 2% paraformaldehyde + 0.05% picric acid in PBS for 60 min at 37°C. Fixed oocytes were permeabilized with PBS containing 0.1% Triton-X100 for 30 min and either immediately labelled or stored at 4°C in PBS + 0.1% Triton-X100 overnight. A blocking solution (PBS + 0.1% Triton-X100 + 0.3% BSA + 150 mM glycine) was used to reduce non-specific secondary antibody binding and the oocytes were washed three times in washing solution (PBS + 0.1% Triton-X100 + 0.3% BSA).

Microtubule and DNA staining

A mouse IgG monoclonal antibody to β-tubulin was employed (Lot No. 053H4806; Sigma; 1:200, 40 min at 37 °C in 100% humidity). After rinsing three times in washing solution at room temperature, a fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (Lot No. 092H4830, Sigma) was used as secondary antibody (1:32, 40 min at 37 °C in 100% humidity). Oocytes stained for β-tubulin were rinsed in washing solution twice and then incubated with 10 μg/ml Hoechst 33258 (Sigma) for 10 min at room temperature. After washing, oocytes were mounted on a glass slide in 50% glycerol containing Slow Fade (Molecular Probe, Eugene, OR, USA). Mounted slides were sealed with clear nail polish and stored at 4°C until analysis.

Statistics

Differences in activation rates between treatment groups were analysed statistically using the χ² test with significance set at P = 0.05.

Results

Activation related changes in oocytes

Changes in microtubule behaviour and nuclear status in monkey oocytes were detected by β-tubulin immunocytochemical and Hoechst 33258 staining respectively and evaluation by fluorescence microscopy. When insemination-derived monkey embryos (2 PN stage-15 h after insemination) were stained for β-tubulin distribution, numerous long, radiating
microtubule bundles were observed throughout the cytoplasm (Figure 1A). Taxol, a microtubule nucleating agent, had no significant effect on this pattern of microtubule distribution. In contrast, in metaphase II (MII) oocytes, tubulin staining was restricted to the spindle region and taxol induced the assembly of numerous asters in the hemisphere opposite the spindle (Figure 1B). Control MII oocytes exposed to 1% DMSO alone did not show aster formation. Chromosomes were displayed on a metaphase plate that stained for β-tubulin, six of the 12 (50%) examined exhibited a presumed sperm centrosome (Figure 1D). Thus, the method employed for sperm head and tail separation apparently resulted in a random but equal retention of the centrosome by the two sperm components.

The oocyte activation rate following sperm head injection (87%) was comparable to that obtained with intact sperm injection (86%; P > 0.05) and both of these activation rates were significantly higher than that seen following sperm tail injection (19%; P < 0.05).

Sperm extract and oocyte activation

In order to examine the possibility that sperm extract contained a soluble factor that is responsible for oocyte activation, we injected 18 mature oocytes with sperm extract (Table I; Figure 1H and I). Seventy-six percent of the successfully injected oocytes became activated as evidenced by the appearance of 1 PN and the absence of taxol-induced microtubule aster formation. Significantly more oocyte activation occurred in sperm extract-injected oocytes than in the sham controls (76% versus 13%; P < 0.05). These results are consistent with the hypothesis that oocyte activation is induced by a factor within spermatozoa that is released after sperm injection.

Oocyte maturity and the activation response

Oocyte maturity is an important factor in determining the oocyte’s response to an activation stimulus. To evaluate the influence of rhesus monkey oocyte maturity on ICSI success, intact immobilized spermatozoa were injected into immature oocytes. Most oocytes (>80%) recovered by follicle aspiration from stimulated animals extrude the first polar body within 10 h of culture. Those oocytes which do not complete maturation in this time frame are presumed to be at the MI stage. Such oocytes, when inseminated, undergo fertilization with a success rate of 41% (83/203; D.P. Wolf, unpublished results). Of the 26 MI oocytes examined for components was performed by micromanipulation, and isolated heads or tails were injected individually into mature oocytes (Table I). Twenty-three oocytes were successfully injected with an isolated sperm head, as confirmed by DNA-specific fluorescence staining of the sperm head, post-injection. Twenty of the 23 (87%) oocytes displayed interphase microtubule patterns indicative of an activated oocyte. Two PN (normal size) were observed in 13 (65%) of these activated oocytes (Figure 1C), while seven (35%) had one (female) PN with a decondensed sperm head. Three oocytes (13%) failed to activate, as evidenced by the presence of an intact MI spindle and a condensed sperm head. Of the 21 MI oocytes surviving after sperm tail injection, three (14%) and one (5%) had 1 or 2 PN respectively, with an interphase microtubular network, and no taxol induced microtubule asters 15 h after the injection, suggesting that these four oocytes were activated. Seventeen of 21 (81%) tail-injected oocytes remained at MII with intact metaphase spindles and numerous taxol-induced microtubule asters, indicating absence of an ooplasmic activation response. Of the non-responding oocytes injected with a tail and stained for β-tubulin, six of the 12 (50%) examined exhibited a presumed sperm centrosome (Figure 1D). Thus, the method employed for sperm head and tail separation apparently resulted in a random but equal retention of the centrosome by the two sperm components.

The oocyte activation rate following sperm head injection (87%) was comparable to that obtained with intact sperm injection (86%; P > 0.05) and both of these activation rates were significantly higher than that seen following sperm tail injection (19%; P < 0.05).

ICSI in the rhesus macaque

We first established that injection of intact, immobilized sperm into mature oocytes resulted in activation and pronuclear formation (Table I). Activation was observed in 19 of 22 (86%) injected oocytes with 12/19 (63%) forming 2 PN within 15 h of injection. Six (32%) of the activated oocytes had 1 PN with a decondensed sperm head and 1 (5%) exhibited 3 PN without an obvious second polar body. Following conventional insemination of nine sibling oocytes, a 100% activation rate was seen with eight out of nine (89%) forming 2 PN and one (11%) forming only 1 PN. They all showed microtubule behaviour characteristic of activated oocytes. To determine whether oocyte activation was induced by mechanical stimulation alone, 16 MII oocytes were subjected to sham injection. Two (13%) showed 1 PN indicating that they were activated; 12 (80%) remained in the metaphase II stage after 15 h of culture. Oocyte activation as induced by sham injection (including mechanical stimulation associated with the injection and transient exposure to high extracellular calcium) occurred at a significantly lower rate (P < 0.05) than that induced by ICSI.
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Figure 1. Epifluorescence photomicrographs depicting microtubule and DNA configurations in rhesus monkey oocytes following insemination, intracytoplasmic injection of intact spermatozoa, isolated sperm heads or sperm tails, or sperm extract. (A) Insemination-derived zygote with two pronuclei (outlined arrows; 15 h post-insemination). Taxol exposure did not initiate microtubular asters; fine radiating microtubule bundles (green) can be seen evenly displayed throughout the cytoplasm (original magnification ×1300). (B) Mature MII oocytes with microtubules associated with a barrel-shaped, second meiotic spindle (outlined arrow). The maternal chromosomes aligned at the equator of the spindle show intense blue staining. Taxol-induced microtubular asters (arrow) were confined to the hemisphere of cytoplasm away from the spindle (original magnification ×1300). (C) Zygote with two pronuclei (outlined arrows) following intracytoplasmic injection of an isolated sperm head, 15 h post-injection. No taxol-induced microtubular asters were present in the cytoplasm. The intense DNA staining at 12 o’clock is from a polar body (original magnification ×1300). (D) Oocyte following intracytoplasmic injection of an isolated sperm tail 15 h post-injection. The oocyte showed no cytoplasmic signs of activation and remained at the MII stage with a metaphase spindle (outlined arrow). A presumptive sperm centrosome, marked with an arrow (original magnification ×2300), is different in intensity and morphology than the taxol-induced asters seen in the hemisphere of cytoplasm opposite the spindle (D-inset; original magnification ×200). (E, F, G) MI oocytes following intact sperm injection without taxol treatment. (E) An injected oocyte arrested in MI, 15 h post-injection (original magnification ×2300). The chromosomes (blue) are aligned at the equator of the centrally positioned, barrel-shaped meiotic spindle (outlined arrow). A condensed spermatozoon (arrow) is located in the cytoplasm. E-inset, lower magnification of the oocyte (original magnification ×200). (F) An injected oocyte which also remained in the MI stage with a spindle (arrow head) but with an injected sperm nucleus that was processed into metaphase. The microtubules (arrow) are associated with the injected sperm head spindle. (G) A zygote derived from intact sperm injection into a MI oocyte (15 h post-injection). The injected spermatozoa induced oocyte activation and transformed the metaphase spindle into a pronucleus (outlined arrow). The arrow marks a decondensed sperm head (original magnification ×1030). (H, I) Oocytes following intracytoplasmic injection of sperm extract. (H) Activated oocyte displaying one pronucleus (outlined arrow) and a microtubule network (original magnification ×1030). (I) Non-activated oocyte exhibiting multiple taxol-induced microtubule asters and metaphase spindle (outlined arrow) (original magnification ×1030).
Discussion

The present results demonstrate that monkey oocytes can be successfully fertilized by microinjection of either intact spermatozoa or of isolated sperm heads. Survival rates of monkey oocytes following microinjection were in the range of 88–94%, which is comparable with human ICSI (87%; Van Steirteghem et al., 1993) but quite different from the results obtained in the mouse (Kimura and Yanagimachi, 1995). This high survival rate may be due to the resiliency of the oocyte plasma membrane.

Mature oocytes from most mammalian species, including primates, are arrested at MII at ovulation. Both meiotic arrest and the condensed state of the oocyte’s chromosomes are maintained by maturation promoting factor (MPF) which is a complex of p34cdc2 kinase and cyclin B. MPF is, in turn, regulated by cytostatic factor (CSF) and the p39 protein product of the c-mos proto-oncogene (Kubiak et al., 1993; Pal et al., 1994). Sperm entry initiates cyclin B degradation and a concomitant decrease in MPF activity leading to the transition from meiotic metaphase to mitotic interphase following oocyte activation. In the rhesus monkey, we have shown that the metaphase/interphase transition is accompanied by an extensive rearrangement of the cytoplasmic microtubule network, and that these changes can be used to monitor cytoplasmic activation. In the unfertilized oocyte, microtubules are short and restricted to the region of the spindle. Furthermore, taxol exposure results in microtubule aster formation throughout the cytoplasm with the exception of the immediate spindle area. In the activated oocyte (zygote), long, relatively stable microtubules are seen that radiate throughout the cytoplasm and were insensitive to taxol exposure. This change is associated temporarily with inactivation of histone H1 kinase (Meng and Wolf, 1995). Verde et al. (1990) correlated the decreased stability of microtubules during metaphase and the lengthening of microtubules in interphase with molecular changes occurring during the cell cycle.

Oocyte activation following fertilization is initiated by sperm-induced changes in intracellular calcium concentrations (Ca²⁺). It should be noted that, in the context of ICSI, mechanical stimulation and exposure to the relatively high extracellular calcium concentration of the medium, induced by sham injection, can alter intracellular Ca²⁺ but do not, alone, cause oocyte activation (Dozortsev et al., 1995 and present report). Similarly, injection of dead spermatozoa does not cause activation (Dozortsev et al., 1995). A repetitive or oscillatory Ca²⁺ stimulus, rather than a single pulse, may be necessary to ensure oocyte activation, since the length of the cell cycle is relatively long in mammals compared with that of the sea urchin or frog (Swann and Lawrence, 1996). Two hypotheses have been presented to account for this Ca²⁺ release after sperm entry. The first invokes a sperm receptor, signal transduction mechanism, in which spermatozoa bind to an oocyte membrane receptor, leading to G-protein and phospholipase C activation and production of the Ca²⁺ releasing agent, inositol 1,4,5-trisphosphate (InsP₃; Miyazaki et al., 1993). A ryanodine-sensitive store of calcium has also been described (Tesarik and Sousa, 1996; Berridge, 1996).

The second hypothesis proposes the existence of a soluble sperm activation (Longo et al., 1986). When spermatozoa fuse with oocytes, an activation factor(s) diffuses into the ooplasm initiating Ca²⁺ release from intra-ooplasmic stores (Swann 1990, 1994; Homa and Swann, 1994). In support of this second hypothesis, an oocyte activation factor has been identified in the cytosolic fraction of rabbit (Stice and Robl, 1990), hamster (Swann 1990), and human sperm (Dozortsev et al., 1995). Recently, Harrington et al. (1996) reported that a Mr 33K sperm cytosolic protein, oscillin, has been identified, cloned, and sequenced. This soluble sperm protein exhibits Ca²⁺ oscillation-inducing activity that may trigger oocyte activation at fertilization in mammals. Immunochemical characterization of oscillin indicates that the protein is concentrated specifically at an intracellular location in the equatorial segment at the precise location where sperm fusion with the oocyte is initiated. In the present studies, we have provided additional evidence in the rhesus monkey for the existence of a soluble sperm factor. Thus, either the entire spermatozoon or the isolated sperm head was required to induce oocyte activation and pronuclear formation, whereas the isolated tail or sham injections were relatively ineffective. Additionally, injection of sperm extracts, at a concentration the equivalent of two to five spermatozoa, induced oocyte activation at rates similar to those following injection of intact spermatozoa or isolated sperm heads.

We observed highly significant differences in zygote formation rates following ICSI of MII versus presumed MI oocytes (63 versus 8%). Part of this difference may reflect the non-

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<th>Sperm component</th>
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<th>Oocyte no.</th>
<th>Lysed no.</th>
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<th>Activateda no. (%)</th>
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<tr>
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aActivated oocytes include those containing 1 PN, 1 PN + decondensed spermatozoa, 2 PN or nucleated blastomeres.
bNon-activated oocytes include those containing MII spindle, MII spindle + condensed sperm, MII spindle + vacuoles or MI spindle + condensed spermatozoo.
viability of oocytes that arrest during meiosis. However, most MI (92%) oocytes that activated and developed a female PN, showed sperm head decondensation but did not develop a male pronucleus. Chian et al. (1992) concluded that, in bovine MI oocytes, oocyte maturation was actually stimulated by sperm penetration. There are three possible explanations for the incomplete activation of rhesus monkey oocytes following sperm injection. The first possibility is that the ICSI procedure bypasses a membrane fusion event that is critical to activation of the immature oocyte. A second possibility is that the intracellular Ca$^{2+}$ release system is not completely developed in the immature oocyte. Finally, either the endoplasmic reticulum, or the ooplasmic Ca$^{2+}$ storage site may not be functionally capable of supporting an activation response. It has been reported that ooplasmic factors are responsible for PN formation (Tesarik and Kopencyn, 1989) and such factors are unstable, with a limited half-life (Borsuk and Tarkowski, 1989). Moreover, breakdown of the sperm nuclear envelope, which is required for PN formation, is also under ooplasmic control and can occur only at the end of MII, although decondensation can occur without nuclear envelope breakdown (Szollosi et al., 1994). The capability of ooplasmic factors to regulate sperm decondensation, protamine–histone exchange and PN formation is dependent on oocyte maturity (Perreault 1992). The disassembly and reorganization of sperm derived structures has recently been examined in rhesus monkey oocytes injected with several spermatozoa (Hewiston et al., 1996; Sutovsky et al., 1996).

In conclusion, the transition from meiotic metaphase to mitotic interphase at fertilization, e.g. oocyte activation, is associated with cytoplasmic changes in microtubule distribution and organization. This cellular marker in the macaque model should be useful in evaluating oocyte maturation, fertilization, and developmental competence, and aid the efforts to understand the mechanism of oocyte activation. We have also extended the ICSI procedure to the rhesus macaque and provided additional evidence for the oscillin hypothesis for sperm-induced oocyte activation. Monkey spermatozoa contain a factor(s) which is required for oocyte activation. The factor(s) is primarily localized in the sperm head and can be recovered from whole sperm extracts. Finally we have demonstrated that oocyte maturity strongly affects the ability of the oocyte to respond to injected spermatozoa.

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Miyazaki, S., Shirakawa, H., Nakada, K. and Honda, Y. (1993) Essential role of the inositol 1, 4, 5- -trisphosphate receptor/Ca$^{2+}$ release system is not completely developed in the immature oocyte. A second possibility is that the intracellular Ca$^{2+}$ release system is not completely developed in the immature oocyte. Finally, either the endoplasmic reticulum, or the ooplasmic Ca$^{2+}$ storage site may not be functionally capable of supporting an activation response. It has been reported that ooplasmic factors are responsible for PN formation (Tesarik and Kopencyn, 1989) and such factors are unstable, with a limited half-life (Borsuk and Tarkowski, 1989). Moreover, breakdown of the sperm nuclear envelope, which is required for PN formation, is also under ooplasmic control and can occur only at the end of MII, although decondensation can occur without nuclear envelope breakdown (Szollosi et al., 1994). The capability of ooplasmic factors to regulate sperm decondensation, protamine–histone exchange and PN formation is dependent on oocyte maturity (Perreault 1992). The disassembly and reorganization of sperm derived structures has recently been examined in rhesus monkey oocytes injected with several spermatozoa (Hewiston et al., 1996; Sutovsky et al., 1996).

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