Analysis of Mouse Oocyte Activation Suggests the Involvement of Sperm Perinuclear Material[1]

Y. Kimura,[3] R. Yanagimachi,[2] S. Kuretake,[3] H. Bortkiewicz,[4] A.C.F. Perry,[5] and H. Yanagimachi

Department of Anatomy and Reproductive Biology, University of Hawaii School of Medicine,
Honolulu, Hawaii 96822

ABSTRACT

The mouse oocyte can be activated by injection of a single, intact mouse spermatozoon or its isolated head. Isolated tails are unable to activate the oocyte. Active sperm-borne oocyte-activating factor(s) (SOAF) appears during transformation of the round spermatid into the spermatozoon. The action of SOAF is not highly species-specific: mouse oocytes are activated by injection of spermatozoa from foreign species, such as the hamster, rabbit, pig, human, and even fish. Some SOAF can be extracted by simple freeze-thawing of (hamster) spermatozoa; additional SOAF is obtained by sequential treatment of spermatozoa with Triton X-100 and SDS. Electron microscopic examination of sperm heads during SOAF extraction suggests that the relatively insoluble SOAF is associated with perinuclear material. When microsurgically injected into oocytes, Triton X-100-treated sperm heads (with perinuclear material, but without any membranes) can activate the oocytes, leading to normal embryonic development. Whereas perinuclear components have been believed to play a purely structural role, these data suggest an additional function for them in oocyte activation.

INTRODUCTION

The fertilizing function of spermatozoa is accomplished by delivering a male genome into an oocyte and activating it, thereby initiating its development into an embryo. The mechanisms by which fertilizing spermatozoa provoke oocyte activation have been the subject of debate from the beginning of this century [1]. Whereas some recent investigators maintain that ligand-receptor interactions between gamete membranes transduce a causative signal, others believe that the spermatozoon activates oocytes by releasing a soluble, oocyte-activating substance into the oocyte (for debate, see [2-6]). The latter notion, proposed by Loeb [7] and a few others in the beginning of this century, has been ignored largely until Dale et al. [8] demonstrated that microsurgical injection of a soluble fraction of sea urchin spermatozoa activates the eggs as efficiently as live spermatozoa. Mammals whose oocytes are activated by injection of (cell-free) sperm extracts include the hamster [9-11], mouse [12], rabbit [12], monkey [13], and human [14, 15]. The present study was initiated to determine 1) the time at which sperm-borne oocyte-activating factor(s) (SOAF) first appears (or becomes biologically

active) during spermatogenesis, 2) the approximate location of SOAF within the mature spermatozoon, and 3) the species-specificity of SOAF. In addition, we undertook a preliminary biochemical characterization of SOAF.

MATERIALS AND METHODS

Reagents

Polyvinyl alcohol (PVA, cold-water-soluble, molecular weight ca. 10 000), soybean trypsin inhibitor (type I-S), Triton X-100, mixed alkyltrimethylammonium bromide (ATAB), SDS, dithiothreitol (DTT), PMSF, benzamidine, Percoll (colloidal solution of polyvinyl pyrrolidone [PVP]-coated silica), and PVP (molecular weight ca. 360 000) were all purchased from Sigma Chemical Co. (St. Louis, MO). Bovine pancreatic trypsin (180 TAME U/mg) and bovine testicular hyaluronidase (200 USP U/mg) were obtained from ICN Biochemicals (Costa Mesa, CA). BSA (fraction V) was purchased from Calbiochem (La Jolla, CA) and mineral oil from Squibb and Sons (Princeton, NJ). All other reagents were obtained from Sigma unless otherwise stated.

Media

The medium used for the culture of mouse oocytes after microsurgery was CZB medium supplemented with 5.56 mM D-glucose [16, 17]. The medium for collection of oocytes from oviducts and subsequent oocyte treatments, including micromanipulation, was a modified CZB (Hepes-CZB) containing 20 mM Hepes-HCl, a reduced amount of $NaHCO_3$ (5 mM), and 0.1 mg/ml PVA instead of BSA. CZB was used under 5% CO_2 in air, and Hepes-CZB was used under air. Isotonic (0.9%) NaCl solutions containing either 1% or 12% (w:v) PVP, referred to as 1% PVP-saline and 12% PVP-saline, respectively, were used to suspend spermatozoa/spermatids/sperm fractions before injection. BM buffer (75 mM NaCl, 24 mM EDTA, and 50 mM Tris-HCl (pH 7.2) [18] was used for treating isolated sperm heads with various reagents before injection. Constituents of the nucleus isolation medium (NIM) were 134 mM KCl, 2.6 mM NaCl, 7.8 mM Na_2HPO_4, 1.4 mM KH_2PO_4, and 0.3 mM PMSF (pH was adjusted to 7.2).

Preparation of Oocytes

B6D2F1 female mice, 8-10 wk old, were induced to superovulate by i.p. injection of 7.5 IU eCG followed by 7.5 IU of hCG 48 h later. Oocytes were collected from oviducts about 15 h after hCG injection. Cumulus cells were removed by a 3-min treatment with 0.1% hyaluronidase in Hepes-CZB. The cumulus-free oocytes were rinsed thoroughly in Hepes-CZB and kept in CZB for up to 3.5 h at 37°C, under 5% CO_2 in air, before injection.

Preparation of Spermatids and Spermatozoa

Seminiferous tubules of B6D2F1 male mice were placed in Hepes-CZB and cut into small pieces with fine scissors. One part of the tubule suspension was mixed with two parts of 12% (w:v) PVP-saline and pipetted vigorously to release spermatozoa/spermatogenic cells from tubules. A small droplet (about 3 µl) of this 8% PVP-containing cell suspension was placed on the bottom of a cooled (16–17°C) microinjection chamber (a plastic Petri dish, 100 mm in diameter and 10 mm in depth), and kept for up to 3 h under mineral oil. Using Nomarski differential interference optics, round spermatids with or without proacrosomal granule/cap (stages 1–7), elongated spermatids (stages 9–12), and fully transformed spermatozoa (stages 15–16) were identified [19] and selected for injection. Mature spermatozoa of the mouse, golden (Syrian) hamster, and rabbit were collected from caudae epididymides. Human and porcine spermatozoa were isolated from freshly ejaculated semen of fertile males. Spermatozoa of fish (swordtail, *Xiphophorus helleri*) and sea urchins (*Tripneustes gratilla*) were collected from the testes of mature males.

In a series of experiments, mouse cauda epididymal spermatozoa were separated into heads and tails before injection. This was achieved by drawing a single spermatozoon, tail first, into the injection pipette until the sperm neck reached the opening of the pipette, then applying a few Piezo pulses [20]. The head and tail were injected separately into different oocytes.

In another series of experiments, isolated sperm heads were treated with various reagents before injection. Mouse or hamster spermatozoa from caudae epididymides were suspended in 5 ml BM buffer and sonicated for 30 sec at 70–80% output of a Biosonik sonicator (Bronwill Scientific, Rochester, NY). Over 95% of the spermatozoa were decapitated by this treatment. The sonicated sperm suspension was centrifuged (700 × g, 5 min), and the pellet was washed with BM buffer and then treated at room temperature with various reagents in NIM or 20 mM Tris-HCl (see footnote of Table 4). The heads were then rinsed thoroughly with 1% PVP-saline, and one part of this suspension was mixed with two parts of 12% PVP-saline. Some sperm heads with or without treatments were fixed for 1 h, at room temperature, with 2% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) and processed for electron microscopy.

Microinjection of Spermatids/Spermatozoa/Sperm Components

Injection of spermatozoa/spermatids and sperm components was performed according to Kimura and Yanagimachi [20, 21]. Spermatozoa from the testis (mouse, fish, and sea urchin), cauda epididymidis (mouse, hamster, and rabbit) and semen (human and pig) were suspended in PVP-saline before injection (see above). A single spermatozoon was drawn, tail first, into the injection pipette until the midpiece-principal piece junction was at the opening of the pipette. Upon application of a few Piezo pulses, the flagellar motion of the spermatozoon ceased [20], perhaps because of a local disintegration/disruption of the plasma membrane. The whole spermatozoon (or isolated head and tail) was injected into an oocyte by using a Piezo-driven pipette [20]. A round spermatid or a transforming spermatid was drawn in and out of the injection pipette repeatedly until its plasma membrane was broken

and the nucleus was separated from the bulk of the cytoplasm. Both the nucleus and cytoplasm were injected together into an oocyte.

In a series of experiments, the ability of Triton X-100-treated mouse spermatozoa to participate in embryonic development was examined. Spermatozoa collected from caudae epididymides were suspended in 3 ml NIM containing 1% (v:v) Triton X-100 and vortexed for 1 min at room temperature. After centrifugation (700 × g, 1 min), spermatozoa were resuspended in Triton-free NIM and centrifuged again (700 × g, 1 min). Then they were resuspended in Triton- and PMSF-free NIM containing 10–12% PVP. Immediately before injection, the sperm head was separated from the tail by applying few Piezo pulses to the sperm-tail junction. The time interval between Triton treatment and sperm head injection was less than 30 min.

Examination of Eggs

The oocytes that had received injections of spermatid nuclei, whole spermatozoa, isolated sperm heads/tails, or chemically treated sperm heads were incubated in CZB for 5–7 h and examined under an inverted microscope for evidence of activation. Some oocytes were fixed and stained [22, 23] to reveal cytological details. Oocytes injected with spermatozoa, spermatids, or isolated sperm heads were considered to have activated normally when each had extruded a second polar body and contained large female and male pronuclei within the ooplasm. In the text and tables, this is denoted by 2PN + 1Pb$_2$. The category "others" (see tabulation) denotes those oocytes exhibiting aberrant fertilization. Some had three or more pronuclei with or without extrusion of the second polar body; others had one large female pronucleus and sperm nuclei either almost intact or only partially decondensed. Oocytes that had received injections of sperm tails or sperm extracts were recorded to have activated normally if they extruded a second polar body and contained one large female pronucleus.

Assessment of the Developmental Ability of Sperm-Injected Oocytes

Normally fertilized pronuclear eggs were cultured in fresh CZB medium. Embryos developing in vitro were examined with the inverted microscope at 24-h intervals for up to 120 h. Some 2-cell embryos were transferred into oviducts of recipient females (Swiss-Webster, albino) that had been mated with males of the same strain during the previous night [20]. Foster mothers were allowed to deliver and raise their own young (red eyes and white coat) as well as to foster pups (black eyes and grey/brown/black coats).

Isolation and Injection of SOAF

We ran preliminary experiments to isolate SOAF by using spermatozoa from hamsters, rather than another species, for three reasons. First, Parrington et al. [24] had previously used hamster spermatozoa to isolate and identify a putative oocyte-activating protein (glucosamine 6-phosphate isomerase or oscillin). Second, hamster and mouse spermatozoa are equally effective in activating mouse oocytes after intracytoplasmic injection (Table 2). Third, the number of spermatozoa collectable from a single hamster cauda epididymidis is > 100 times higher than

that from a single mouse cauda epididymidis. We used two methods to isolate SOAF.

Method 1. Two caudae epididymides were isolated from a mature golden hamster. After blood adhering to their surfaces was removed carefully, the proximal two-thirds of each epididymis was wrapped with tissue paper (Kimwipes; Kimberly-Clark, Atlanta, GA). While finger pressure was applied through the tissue paper, the distal epididymis was punctured with a sharp needle or forceps to allow a dense sperm mass to ooze out. This was repeated several times until almost all of the spermatozoa in the distal cauda epididymidis were collected. Spermatozoa were suspended for 3 min in 30 ml of erythrocyte-lysing buffer (155 mM NH_4Cl, 10 mM $KHCO_3$, 2 mM EDTA; pH 7.4) before centrifugation (1500 $\times$ g, 5 min). The sperm pellet was resuspended in 30 ml of Dulbecco's PBS and centrifuged (1500 $\times$ g for 5 min). The supernatant was discarded, and the pellet, containing approximately 2 $\times$ 10^8 spermatozoa, was suspended in 0.5 ml PBS in a polypropylene centrifuge tube. The tube was placed in liquid nitrogen for 10 min, then warmed up to room temperature, by using running tap water. Death of all spermatozoa was confirmed by staining with propidium iodide/SYBR 14 (live/dead Ferti-Light sperm viability kit; Molecular Probes Inc., Eugene, OR). The preparation was centrifuged (22 500 $\times$ g) for 30 min at 4°C. Part of the supernatant was concentrated by using Amicon filters (molecular weight cutoff 10 000, 30 000, 50 000, or 100 000; Amicon Division, W.R. Grace & Co., Beverly, MA) and centrifuged (3800 $\times$ g, 10 min) to collect filtrates. Approximately 5 pl each of the unfractionated and fractionated sperm extracts was injected into individual oocytes.

Method 2. Spermatozoa from two caudae epididymides of a mature hamster male were collected and treated for 3 min with erythrocyte-lysing buffer at room temperature. Subsequent treatments were performed at 4°C whenever possible. The sperm pellet was suspended in 30 ml YM medium (0.9% NaCl containing 1 mM PMSF, 1 mM benzamidine, and 2.5 mM EDTA; 10 mM Tris-HCl supplemented with 1% [v:v] Triton X-100) (pH 7.2). Unless specifically stated, all media and water mentioned below were buffered with 10 mM Tris-HCl to pH 7.0–7.4.

Spermatozoa in the 1% Triton-containing YM medium were sonicated until > 98% sperm heads and tails were separated. A 15-ml aliquot of the sonicated sperm suspension was placed gently on 30 ml of 90% Percoll solution in YM. After centrifugation at 22 500 $\times$ g for 30 min, sperm heads at the bottom of the centrifuge tube were collected. After being washed once by centrifugation (1500 $\times$ g, 10 min) with YM medium containing 1% Triton, sperm heads were resuspended in 2–4 ml of 1% (w:v) SDS in distilled water for 10 min with occasional agitation. After centrifugation (1500 $\times$ g for 10 min), the

supernatant was collected, diluted with distilled water to 20 ml, and filtered through a 0.45-μm pore filter (Millipore Corp., Bedford, MA). Before filtration, 1 drop (about 20 mg) of Percoll was added to the 20-ml supernatant solution. The solution was dialyzed successively against 1) distilled water overnight; 2) 3% Triton X-100 in distilled water for 4–5 h; 3) running tap water (pH 7.5), room temperature, for 5–10 min; and 4) nonbuffered distilled water (pH 5.2) for 1–2 days, with the distilled water renewed at frequent intervals. The solution was freeze-dried to allow SOAF to be adsorbed onto drying Percoll. Some solution was heated (90°C) for 30 min before freeze-drying. Freeze-dried material (Percoll) was kept at -50°C before injection into oocytes.

Gel Electrophoresis

Crude extracts of hamster cauda epididymal spermatozoa independently prepared by methods 1 and 2 were analyzed by SDS-PAGE on a 10% (w:v) polyacrylamide gel. After electrophoresis, proteins were transferred to a solid support by using a standard Western blot protocol and were probed with a polyclonal guinea pig antibody raised against mouse calicin [25]. After the filter was washed, primary antibody binding sites were labeled with peroxidase-conjugated, anti-guinea pig, rabbit IgG (the second antibody) and were detected after the chromogenic breakdown of 3,3'-diaminobenzidine.

RESULTS

Oocyte-Activating Capacity of Mouse Spermatozoa and Spermatids

Table 1 summarizes the results of experiments in which mouse testicular spermatozoa and spermatids were injected into mouse oocytes. Testicular spermatozoa activated oocytes very efficiently, whereas round spermatids (stages 1–6) failed to do so. Spermatids in the process of transformation into spermatozoa (stages 9–11) occasionally could activate the oocytes.

Spermatozoa of foreign species also could activate mouse oocytes (Table 2). Hamster and rabbit spermatozoa, for example, activated mouse oocytes very efficiently. However, although these activated oocytes first appeared intact, they began to deform after extrusion of the second polar body (Fig. 1) and never reached the 2-cell stage. Such deformation did not occur when hamster and rabbit spermatozoa had been sonicated and sperm heads without acrosomal caps were injected (Fig. 2). Unlike hamster and rabbit spermatozoa, intact human spermatozoa activated mouse oocytes without deforming oocytes. Sonicated pig spermatozoa activated mouse oocytes. A single fish spermatozoon activated only about 20% of mouse oocytes. However, the activation rate increased to 50% when three

TABLE 1. Comparison of the oocyte-activating abilities of testicular spermatozoa, round spermatids, and transforming spermatids of the mouse in oocytes examined 5–7 h after injection.

Cells injected	Total no. of oocytes injected (no. exp.)	No. (%) of surviving oocytes	No. (%) of activated oocytes among surviving oocytes	No. (%) of activated eggs with:	
				2 PN + Pb$_2$	Others
Testicular sperm	80 (3)	71 (89)	71 (100)	71 (100)	0 (0)
Round spermatids	50 (3)	46 (92)	0 (0)	—	—
Elongated spermatids	50 (3)	46 (92)	27 (59)	23 (85)	4 (15)[a]
None[b] (Sham)	85 (7)	81 (95)	2 (2)	0 (0)	2 (100)[a]

[a] These 6 eggs each had 1 PN + 1 Pb$_2$.
[b] Approximately 30 pl of 12% PVP-saline was injected.

TABLE 2. Response of mouse oocytes injected with spermatozoa of foreign species examined 5–7 h after sperm injection.

Species	Special treatment prior to injection	Total no. of oocytes injected	No. (%) surviving oocytes	No. (%) of activated oocytes among surviving	No. (%) of activated eggs with	
					2 PN + 1 P$_2$	Others
Human	—	40	33 (83)	29 (88)	28 (97)	1 (3)[d]
Hamster	—	28	26 (93)	26 (100)	0 (0)	26 (100)[e]
	Sonicated	23	19 (83)	19 (100)	18 (95)	1 (5)[d]
Rabbit	—	31	23 (74)	22 (96)	2 (9)	20 (91)[e]
	Sonicated	31	31 (100)	31 (100)	31 (100)	0 (0)
Pig	Sonicated	21	16 (76)	16 (100)	5 (31)	11 (69)[d]
Fish (Swordtail)	—[a]	38	34 (89)	7 (21)	6 (86)	1 (14)[d]
	—[b]	15	14 (93)	7 (50)	0 (0)	7 (100)[d]
Sea urchin (T. gratilla)	—[c]	16	16 (100)	0 (0)	0 (0)	0 (0)

[a] A single spermatozoon was injected into each oocyte.
[b] Three spermatozoa were injected into each oocyte.
[c] One to three spermatozoa were injected into each oocyte.
[d] These include zygotes with 1 or 3 PN, with or without Pb$_2$, as well as eggs with a partially decondensed nucleus.
[e] All of these eggs were severely deformed and, because of this, cytological details were difficult to determine, but they were at either telophase II or the pronuclear stage.

fish spermatozoa were injected into each oocyte. Sea urchin spermatozoa (1–3 per oocyte) failed to activate mouse oocytes (see *Note added in Proof*).

## Oocyte-Activating Capacity of Mouse Sperm Head and Tail

Mouse cauda epididymal spermatozoa were separated into heads and tails by applying a few Piezo pulses to the sperm-tail junction (neck) of motile spermatozoa immediately before injection. A single sperm head or a single tail was injected into each oocyte. Sperm heads activated oocytes very efficiently, whereas tails failed to do so above the negative control level (Table 3). Similarly, isolated human sperm heads activated mouse oocytes, but isolated tails did not, even when 2–3 tails were injected into an oocyte (data not shown).

Some Properties of Triton X-100-Insoluble SOAF

To gain insights into the nature of SOAF, isolated mouse and hamster sperm heads were treated with various reagents before injection into oocytes (Table 4). Judged by its ability to activate oocytes, a proportion of SOAF appeared to be resistant to Triton treatment, but vulnerable to treatment with either trypsin, SDS, or ATAB-DTT treatments. Light and electron microscopic examination of mouse sperm heads revealed that Triton treatment removed all membranes, but left perinuclear material around the nucleus (Figs. 3A and 4A). Trypsin treatment removed or altered perinuclear materials extensively (Fig. 4B). Treatments of mouse spermatozoa with Triton and SDS removed most of perinuclear materials (Figs. 3B and 4C). Simultaneous treatment with DTT and ATAB not only removed perinuclear materials, but also caused partial decondensation of the nucleus (Fig. 4D).

A crude hamster sperm extract obtained by method 1, described in the *Materials and Methods*, activated mouse oocytes very efficiently (Table 5). Membrane filtration suggests that the molecular weight of the active component(s) is between 30 000 and 100 000. Gel electrophoresis of the crude extract revealed more than 20 components ranging from molecular weight of 30 000 to 120 000 (Fig. 5A).
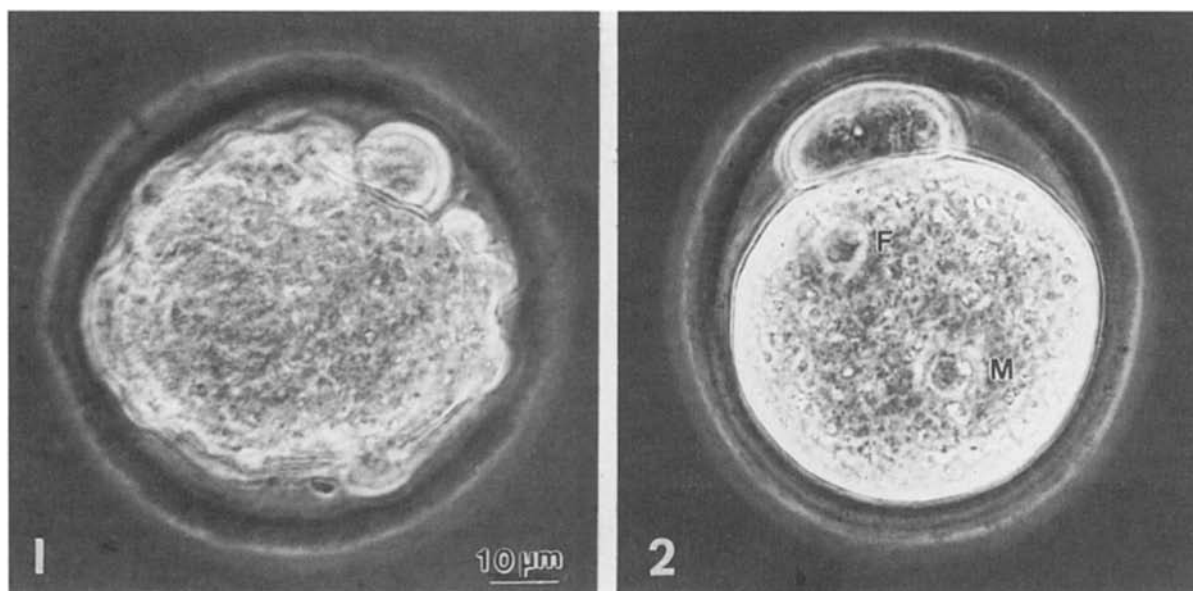
FIGS. 1 AND 2. Phase-contrast micrographs of mouse oocytes, 5–6 h after sperm injection. 1) An oocyte injected with an acrosome-intact hamster spermatozoon; note an irregular shape of the vitellus. 2) An oocyte injected with a sonicated hamster spermatozoon without acrosomal cap; male (M) and female (F) pronuclei are seen within normal-looking vitellus.

TABLE 3. Oocyte-activating ability of the head and tail of mouse spermatozoon.

Sperm fragment injected	Total no. of oocytes injected (no. exp.)	No. (%) of oocytes surviving	No. (%) of activated oocytes among surviving	No. (%) of activated eggs with:	
				2 PN + 1 Pb$_2$	Others
Head	184 (9)	157 (85)	155 (99)	143 (92)	12 (8)[b]
Tail	54 (5)	51 (94)	1 (n2)	0 (0)	1[c]
Control[a]	65 (6)	63 (97)	2 (3)	0 (0)	2[c]

[a] Approximately 30 pl of 12% PVP-saline was injected.
[b] These eggs had 1 or >3 PN with or without Pb$_2$.
[c] These eggs had 1 PN and Pb$_2$.

The amount of freeze-dried material extracted from 10^8 SDS-treated sperm heads by method 2 was too little for accurate weight determination. In an attempt to stabilize these small quantities, Percoll was added to the hamster sperm head extract before freeze-drying (method 2) so that extracted materials were adsorbed onto it. Percoll crystals had various sizes and shapes, many of which were rod-shaped and very flexible (Fig. 6). They did not dissolve in isotonic solutions of NaCl or KCl. Injection of a freeze-dried Percoll crystal into an oocyte was easy, but the precise amount of SOAF injected could not be determined. Nevertheless, 95% of 42 oocytes were activated when freeze-dried, hamster SOAF-adsorbed Percoll crystals were injected (Fig. 7 and Table 6). In contrast, none of the 51 oocytes were activated after injection of Percoll crystals alone. Similarly, Percoll crystals with preheated hamster SOAF could not activate mouse oocytes (Table 6). Gel electrophoresis of SDS-extract from Triton-X-100-treated hamster sperm heads (Fig. 5B) revealed some 12 major components after staining with Coomassie blue dye. Western blot analysis (Fig. 5C) revealed that calicin is a constituent of this extract.

TABLE 4. Effects of various treatments at 25°C of isolated heads of mature epididymal spermatozoa on their ability to activate mouse oocytes.

Sperm heads treated with	Total no. of oocytes injected	No. (%) of oocytes		No. (%) of activated eggs with	
		Surviving	Activated	2 PN + 1 Pb$_2$	Others[f]
Mouse					
None (control)	31 (3)	26 (84)	26 (100)	24 (92)	2 (8)
1% Triton[a]	46 (3)	41 (89)	41 (100)	42 (95)	2 (5)
0.1% Trypsin[b]	42 (3)	38 (90)	0 (0)	—	—
1% SDS[c]	51 (3)	45 (88)	1 (2)	0 (0)	—
Triton-SDS[d]	43 (3)	39 (90)	0 (0)	—	—
ATAB-DTT[e]	29 (3)	24 (83)	0 (0)	—	—
Hamster					
1% Triton	18 (2)	16 (89)	16 (100)	15 (94)	1 (6)
0.1% Trypsin[a]	20 (2)	19 (95)	1 (5)	1 (100)	0 (0)
1% SDS[c]	20 (2)	19 (95)	0 (0)	—	—
Triton-SDS[d]	24 (2)	21 (87)	0 (0)	—	—

[a] 5-min treatment with 1% Triton X-100 in NIM.
[b] 5-min treatment with 0.1% trypsin in NIM.
[c] 5-min treatment with 1% SDS in 20 mM Tris-HCl (pH 7.2).
[d] 5-min treatment with 1% Triton X-100 in NIM followed by 5-min treatment with 1% SDS in 20 mM Tris-HCl (pH 7.2).
[e] 10-min treatment with 0.5% ATAB plus 2 mM DTT in 20 mM Tris-HCL (pH 8.2).
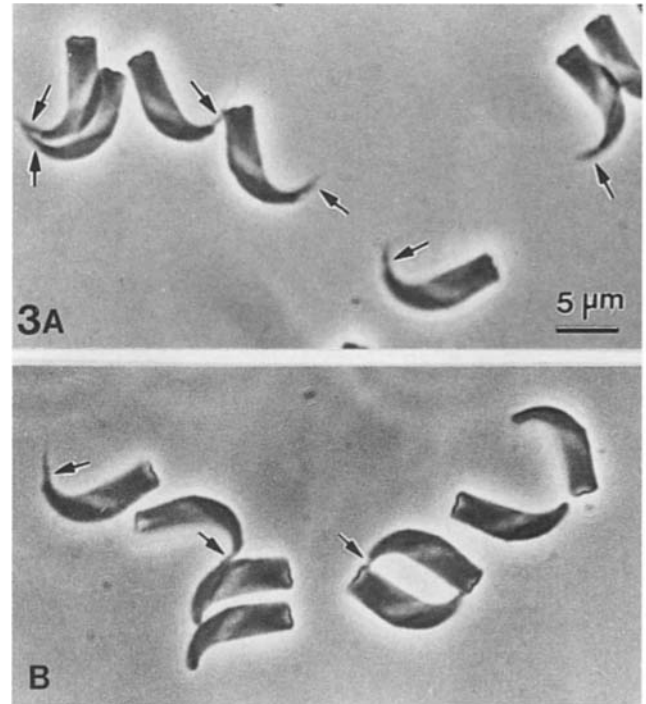[f] These include eggs with 1 or 3 PN with or without Pb$_2$.

FIG. 3. Phase-contrast micrographs of hamster sperm heads treated with A) Triton X-100 or B) Triton X-100 plus SDS. Arrows indicate the perforatorium, a frontal accumulation of the perinuclear material. Note that the perforatorium is absent in many sperm nuclei in B.

Developmental Ability of Mouse Oocytes Injected with Triton-Treated Mouse Sperm Heads

When Triton-treated and intact (control) mouse sperm heads were injected into mouse oocytes, there was no difference between these two groups with respect to the time and rate of oocyte activation, the rate of normal pronuclei development (Table 7), and the rates of normal preimplantation and postimplantation development (Tables 8 and 9).

TABLE 5. Activation of mouse oocytes injected with supernatant from hamster spermatozoa prepared by Method 1 (see Materials and Methods).

Sperm extract	Total no. of oocytes injected (no. exp.)	No. of surviving oocytes	No. (%) activated eggs
Unfractioned	15 (2)	14	14 (100)
Fraction			
<10 000 M$_r$	10 (2)	10	0 (0)
<30 000 M$_r$	10 (2)	10	0 (0)
<100 000 M$_r$	16 (3)	15	15 (100)

TABLE 6. Activation of mouse oocytes injected with partially purified hamster SOAF freeze-dried before injection.

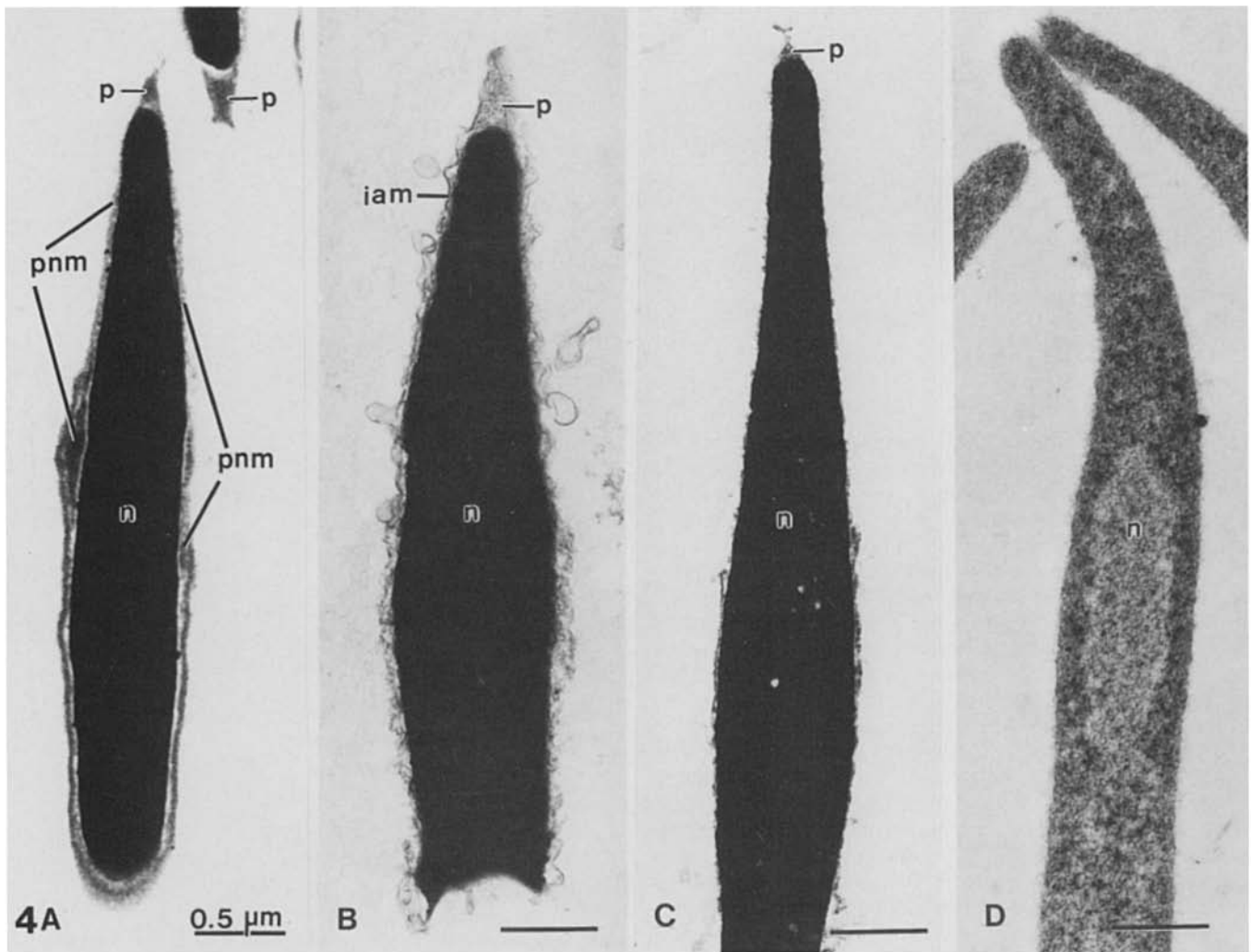| SOAF heat-treated (90°C, 30 min) before freeze-drying	Total no. oocytes injected (no. exp.)	No. of surviving oocytes	No. (%) of activated eggs among those surviving
−	42 (4)	40 (95)	40 (100)
+	23 (2)	21 (91)	0 (0)

FIG. 4. Sagittal sections of mouse sperm heads treated with A) Triton X-100, B) trypsin, C) Triton X-100 and SDS, and D) ATAB and DTT. iam, Inner acrosomal membrane; n, nucleus; pnm, perinuclear material; p, perforatorium = apical accumulation of perinuclear material.

DISCUSSION

The results of the present study support the notion that mammalian spermatozoa introduce some substances into oocytes to activate them. In the mouse, SOAF is(are) lo-
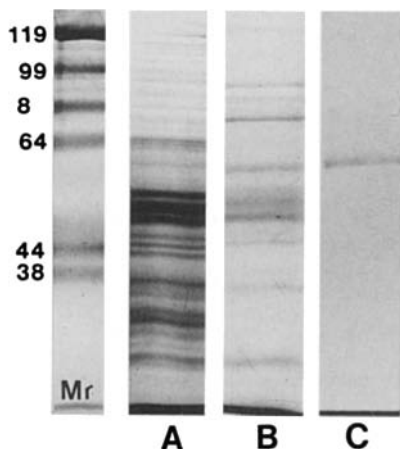

FIG. 5. SDS-PAGE of A) crude whole sperm extract obtained by method 1, B) sperm head extract obtained by method 2 (see *Materials and Methods*). C) SDS-PAGE immunoblot of sperm head extract (B) with anti-calicin antibody.

cated within the sperm head, not in the tail (Table 3). It has already been reported that isolated human and monkey sperm heads can activate oocytes after injection [13, 26]. In humans, injection of round spermatids activates human oocytes [27, 28], indicating that these cells contain SOAF activity. In contrast, round spermatids of the mouse are unable to do so until they begin to elongate (Table 1). Thus, synthesis or activation of mouse SOAF seems to occur during spermiogenesis. It is interesting to note that the intracellular assembly of the perinuclear material is initiated and completed during spermiogenesis [29–31]. Round-headed human spermatozoa with incomplete assembly of the perinuclear materials [32, 33] are unable to activate oocytes when injected [34, 35].

TABLE 7. Response of mouse oocytes to intact and Triton-treated spermatozoa, examined between 5 and 7 h after injection.

Sperm injected	No. of exp.	Total no. oocytes injected	No. (%) oocytes surviving	No. (%) surviving eggs normally fertilized
Intact	5	59	50 (84.7)	50 (100)
Triton-treated*	3	29	25 (86.2)	25 (100)

* Mouse spermatozoa in NIM medium containing 1% Triton-X-100 were vortexed for 1 min, then washed by centrifugation (see *Materials and Methods*).
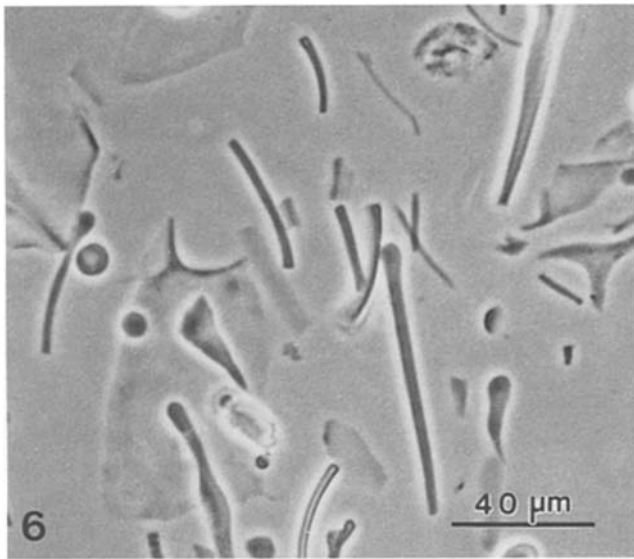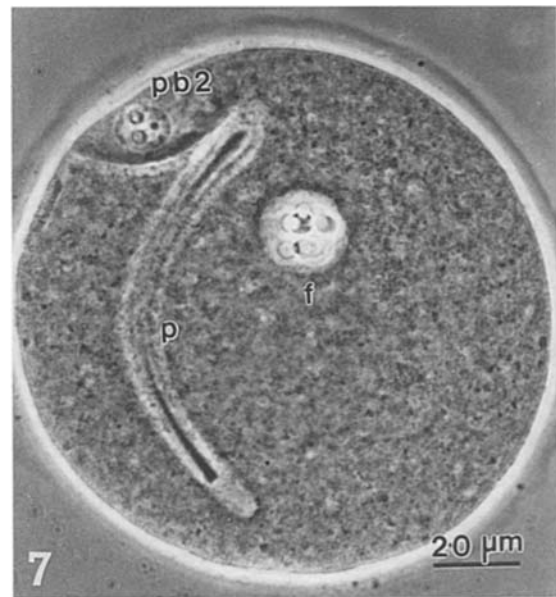
FIG. 6. Percoll crystals with adsorbed SOAF.

FIG. 7. Mouse oocyte activated by injection of a Percoll crystal with adsorbed SOAF. f, Female pronucleus; p, Percoll crystal; pb2, the second polar body.

The action of SOAF is not highly species-specific: spermatozoa from the hamster, rabbit, pig, human, and even fish activate mouse oocytes when injected (Table 2). This apparent lack of species specificity of SOAF is consistent with previous reports of sperm extracts activating oocytes in homologous and heterologous species [10, 12, 14, 15, 36].

It should be noted that although mouse, hamster, rabbit, pig, and human spermatozoa were all capable of activating mouse oocytes (Table 2), injection of acrosome-intact hamster and rabbit spermatozoa is detrimental to mouse oocytes. The oocytes deformed extensively, starting several hours after injection, and never reached the 2-cell stage. The "cytotoxicity" of hamster and rabbit spermatozoa disappeared when they were sonicated and acrosomeless sperm heads were injected into oocytes. Since acrosomes of hamster and rabbit spermatozoa are much larger than those of mouse and human spermatozoa, we first thought that introduction of a relatively large volume of acrosomal contents was detrimental. However, we abandoned this idea because injection of acrosome-intact hamster spermatozoa into hamster oocytes did not produce any noticeable detrimental effects. Since hamster and mouse oocytes have approximately the same volume, it is perhaps the tolerance of the ooplasm to exotic acrosomal contents, not just the volume of acrosomal contents introduced, that determines the level of acrosomal toxicity.

One candidate sperm-borne oocyte-activating component, isolated from hamster spermatozoa, was called oscillogen (and later oscillin) by Swann [4, 37] because it induces repetitive intracellular free calcium ($[Ca^{2+}]_i$) elevations within the oocyte when injected. Normal fertilization,

direct intracytoplasmic sperm injection (ICSI), and injection of sperm extracts all cause such repetitive $[Ca^{2+}]_i$ elevation in various species, including the human [14, 38, 39], cattle [40, 41], rabbit [42, 43], hamster [9, 10, 44, 45], and mouse [10, 11, 46]. Such $[Ca^{2}]_i$ elevations are believed to trigger both cortical granule exocytosis and the resumption of oocyte meiosis through as yet undefined mechanisms (for discussion, see [5, 47, 48]).

According to Swann [9], hamster and porcine oscillogens are trypsin-sensitive protein complexes of $M_r >$ 100 000. The oocyte-activating factors isolated from rabbit spermatozoa are also trypsin-sensitive [12], and that of human spermatozoa is heat-labile [15]. In our study on the hamster, both crude extract from whole spermatozoa and partially purified SOAF from isolated sperm heads were capable of activating mouse oocytes when injected (Tables 5 and 6). Our experiments showed that Triton-treated sperm heads possessed oocyte-activating ability, whereas SDS-treated sperm heads did not (Table 4). The most notable difference between these two types of sperm heads was the presence or absence of perinuclear material. It was present in Triton-treated sperm heads (Fig. 4A) and was almost absent in Triton-/SDS-treated heads (Fig. 4C). Triton treatment removed all plasma and acrosomal membranes as well as acrosomal contents. Oscillin is localized specifically in the equatorial region of the sperm head [24]. Since Triton removes all non-nuclear components of the equatorial region of the sperm head except for the perinuclear material,

TABLE 8. In vitro development of mouse eggs fertilized by injection of intact and Triton-treated spermatozoa.

Sperm injected	Total no. of eggs cultured (No. of exp.)	No. (%) eggs developed into			
		2-cell	4-cell	Morula	Blastocyst
Intact*	119 (6)	118 (99)	115 (97)	108 (91)	81 (68)[a]
Triton-treated	25 (3)	25 (100)	25 (100)	20 (80)	19 (76)[b]

* Cited from Kimura and Yanagimachi [20].
[a-b] $p > 0.1$, using chi-square test.

TABLE 9. Term development of embryos derived from the eggs fertilized by injection of intact and Triton-treated spermatozoa.

Sperm	No. of embryos transferred	No. of recipients	No. (%) of live offspring developed from transferred embryos
Intact*	106	8	32 (30)[a]
Triton-treated	69	8	19 (28)[b]

* Cited from Kimura and Yanagimachi [20].
[a-b] $p > 0.1$, using chi-square test.

it is very unlikely that the "insoluble" SOAF residing in/with the perinuclear material is identical with the "readily soluble" oscillin. However, it is possible that oscillin released from spermatozoa during extraction procedures binds to the perinuclear material. This point must be clarified by future investigations.

Among extra-nuclear mammalian sperm proteins, thecin [49] and calicin [25, 50–52] deserve attention. Thecin (M_r 75 000, 77 000, and 80 000) denotes proteins from sperm perinuclear theca; calicin (M_r 60 000) refers to a protein in the posterior calyx of the sperm head. Since thecin apparently is not extractable by 1% SDS treatment but is extracted by a subsequent CTAB/DTT treatment, it alone cannot account for the SOAF activity we reported here. Calicin is similar to the SOAF in that it is resistant to Triton treatment. Calicin [50, 51] and SOAF are present in the spermatozoa of many different mammalian species. Calicin, SOAF, and thecin all appear (become biologically active) or are assembled in their final forms during spermiogenesis ([25, 29] and see Table 1 of this paper). It is important to note that calicin and thecin are just two of a large number of proteins constituting the perinuclear material [25, 52].

Distribution of calicin is restricted to the postacrosomal region. Since the perinuclear material in this region of the sperm head mingles quickly with the ooplasm after sperm-oocyte membrane fusion [48, 53], it is tempting to speculate that calicin represents the SOAF. Longo et al. [25] speculated that both calicin and other perinuclear proteins contribute to the formation of perinuclear theca, which functions as 1) an architectural element involved in the shape changes of the nucleus during spermiogenesis and 2) a cement linking the nucleus with the overlying plasma membrane when spermiogenesis is completed. We propose here that another important function of the perinuclear material is to activate oocytes.

Calicin is water-insoluble [25]. Similarly, the SOAF is not readily soluble in ordinary salt solutions (including oocyte culture medium and intracellular buffer). However, inside the sperm cell, a part of the SOAF may exist in a readily soluble form. It has been known for many years that perinuclear material dissolves readily by SDS-DTT treatment [54]. The fact that the perinuclear material in the postacrosomal region rapidly disperses into the ooplasm soon after sperm-oocyte fusion [48, 53] may be accounted for by drastic changes due to its exposure to the ooplasm. Although oocyte activation can be triggered by ligand-receptor interactions between gamete membranes [3, 6] or oscillin [4, 55], one should remember that Triton-treated mouse spermatozoa without any membranes (including plasma and acrosomal membranes) and "cytosol" are fully capable of activating and fertilizing oocytes ([56], and Table 7), culminating in the birth of normal offspring (Table 9).

NOTE ADDED IN PROOF

We later found that 21.4% of mouse oocytes are activated when 10 sea urchin spermatozoa are injected into each mouse oocyte (Wakayama, Uehara, Hayashi and Yanagimachi. The response of mouse oocytes injected with sea urchin spermatozoa; Zygote 1997; 5:229–234).

ACKNOWLEDGMENT

REFERENCES

1. Lillie FR. Problems of Fertilization. Chicago: University of Chicago Press: 1919.
2. Whitaker M, Swann K. Lighting the fuse at fertilization. Development 1993; 117:1–12.
3. Foltz KR, Schilling FM. Receptor-mediated signal transduction and egg activation. Zygote 1993; 1:276–279.
4. Swann K. The soluble sperm oscillogen hypothesis. Zygote 1993; 1:273–279.
5. Swann K, Ozil P. Dynamics of the calcium signal that triggers mammalian egg activation. Int Rev Cytol 1994; 152:183–222.
6. Schultz RM, Kof GS. Molecular basis of mammalian egg activation. Curr Top Dev Biol 1995; 30:21–62.
7. Loeb J. The Organism as A Whole from a Physicochemical Viewpoint. New York: G.P. Putnam's Sons; 1916.
8. Dale B, De Felice LJ, Ehrenstein G. Injection of a soluble sperm fraction into sea-urchin eggs triggers the cortical reaction. Experientia 1985; 41:1068–1070.
9. Swann K. A cytosolic sperm factor stimulates repetitive calcium increases and mimics fertilization in hamster eggs. Development 1990; 110:1295–1302.
10. Swann K. Different triggers for calcium oscillations in mouse eggs involve a ryanodine-sensitive calcium store. Biochem J 1992; 287:79–84.
11. Swann K. Ca^{2+} oscillations and sensitization of Ca^{2+} release in unfertilized mouse eggs injected with a sperm factor. Cell Calcium 1994; 15:331–339.
12. Stice SL, Robl JM. Activation of mammalian oocytes by a factor obtained from rabbit sperm. Mol Reprod Dev 1990; 25:272–280.
13. Meng L, Wolf DP. Sperm-induced activation in the Rhesus monkey: nuclear and cytoplasmic changes following intracytoplasmic sperm injection. Hum Reprod 1997; 12:1062–1068.
14. Homa ST, Swann K. A cytosolic sperm factor triggers calcium oscillations and membrane hyperpolarizations in human oocytes. Hum Reprod 1994; 9:2356–2361.
15. Dozortsev D, Rybouchkin A, De Sutter P, Qian, C, Dhont M. Human oocyte activation following intracytoplasmic injection: the role of the sperm cell. Hum Reprod 1995; 10:403–407.
16. Chatot CL, Ziomek A, Bavister BD, Torres I. An improved culture medium supports development of random-bred 1-cell mouse embryo. J Reprod Fertil 1989; 86:7679–7688.
17. Chatot C, Torres I, Ziomek CA. Development of 1-cell embryos from different strains of mice in CZB medium. Biol Reprod 1990; 42:432–440.
18. Bellve AR, Chandrika R, Martinova YS, Barth AH. The perinuclear matrix as a structural element of the mouse sperm nucleus. Biol Reprod 1992; 47:451–465.
19. Oakberg EF. Description of spermatogenesis in the mouse and its use in analysis of the cycle of the seminiferous epithelium and germ cell renewal. Am J Anat 1956; 99:391–413.
20. Kimura Y, Yanagimachi R. Intracytoplasmic sperm injection in the mouse. Biol Reprod 1995; 52:709–720.
21. Kimura Y, Yanagimachi R. Mouse oocytes injected with testicular spermatozoa or round spermatids can develop into normal offspring. Development 1995; 121:2397–2405.
22. Perreault SD, Zirkin BR. Sperm nuclear decondensation in mammals: role of sperm-associated proteinase in vivo. J Exp Zool 1982; 224:253–257.
23. Yanagida K, Yanagimachi R, Perreault SD, Kleinfeld RG. Thermostability of sperm nuclei assessed by microinjection into hamster oocytes. Biol Reprod 1991; 44:440–447.
24. Parrington J, Swann K, Shevchenko VL, Sesay AK, Lai FA. Calcium oscillations in mammalian eggs triggered by a soluble sperm protein. Nature 1996; 379:364–368.
25. Longo FJ, Krohne G, Franke WW. Basic proteins of the perinuclear theca of mammalian spermatozoa and spermatids: a novel class of cytoskeletal elements. J Cell Biol 1987; 105:8105–1120.
26. Bourne H, Richings N, Liu DY, Clarke GN, Harari O, Baker HW. Sperm preparation for intracytoplasmic injection: methods and relationship to fertilization results. Reprod Fertil Dev 1995; 7:177–184.
27. Tesarik J, Mendoza C, Testart J. Viable embryos from injection of round spermatids into oocytes. New Engl J Med 1995; 333:525.
28. Tesarik J. Fertilization of oocytes by injecting spermatozoa, spermatids and spermatocytes. Rev Reprod 1996; 1:149–152.
29. Bellve AR, Chandrika R, Barth A. Temporal expression, polar distri-

bution and transition of an epitope domain in the perinuclear theca during mouse spermatogenesis. J Cell Sci 1990; 96:745–756.

30. Oko RJ. Developmental expression and possible role of perinuclear theca proteins in mammalian spermatozoa. Reprod Fertil Dev 1995; 7:777–797.

31. Oko R, Maravel D. Distribution and possible role of perinuclear theca proteins during bovine spermatogenesis. Micr Res Tech 1995; 32:520–532.

32. Kullander S, Rausing A. On round-headed human spermatozoa. Int J Fertil 1975; 20:33–40.

33. Escalier D. Failure of differentiation of the nuclear-perinuclear skeletal complex in the round-headed human spermatozoa. Int J Dev Biol 1990; 34:287–297.

34. Rybouchkin A, Dozortsev D, Pelinck MJ, De Sutter P, Dhont M. Analysis of the oocyte activating capacity and chromosomal complement of round-headed human spermatozoa by their injection into mouse oocytes. Hum Reprod 1996; 11:2170–2175.

35. Battaglia DE, Koehler JK, Klein NA, Tucker MJ. Failure of oocyte activation after intracytoplasmic sperm injection using round-headed sperm. Fertil Steril 1997; 68:118–122.

36. Rybouchkin A, Dozortsev D, de Sutter P, Qian C, Dhont M. Intracytoplasmic injection of human sperm into mouse oocytes: a useful model to investigate the oocyte-activating capacity and the karyotype of human spermatozoa. Hum Reprod 1995; 10:1130–1135.

37. Swann K. Soluble sperm factors and Ca^{2+} release in eggs at fertilization. Rev Reprod 1996; 1:33–39.

38. Tylor CT, Lawrence YM, Kingsland CR, Biljan MM, Cuthbertson KSR. Oscillations in intracellular free calcium induced by spermatozoa in human oocytes at fertilization. Hum Reprod 1993; 8:2174–2179.

39. Tesarik J, Sousa M, Testart J. Human oocyte activation after intracytoplasmic sperm injection. Hum Reprod 1994; 9:511–518.

40. Sun FZ, Bradshaw JP, Galli C, Moor RM. Changes in intracellular calcium concentration in bovine oocytes following penetration by spermatozoa. J Reprod Fertil 1994; 101:713–719.

41. Nakada K, Mizuno J, Shiraishi K, Endo K, Miyazaki S. Initiation, persistence, and cessation of a series of intracellular Ca^{2+} responses during fertilization of bovine eggs. J Reprod Dev 1995; 41:77–84.

42. Fissore RA, Robl JM. Sperm, inositol triphosphate, and thimerosal-induced intracellular Ca^{2+} elevations in rabbit eggs. Dev Biol 1993; 159:122–130.

43. Fissore RA, Robl JM. Mechanisms of calcium oscillations in fertilized rabbit eggs. Dev Biol 1994; 166:634–642.

44. Miyazaki S, Hashimoto N, Yoshimoto Y, Kishimoto T, Igusa Y, Hiramoto Y. Temporal and spatial dynamics of the periodic increase in intracellular free calcium at fertilization of golden hamster eggs. Dev Biol 1986; 118:259–267.

45. Miyazaki S, Shirakawa H, Nakada K, Honda Y. Essential role of the inositol 1,4,5-triphosphate receptor/Ca^{2+} release channel in Ca^{2+} waves and Ca^{2+} oscillations at fertilization of mammalian eggs. Dev Biol 1993; 158:62–78.

46. Hehlmann LM, Kline D. Regulation of intracellular calcium in the mouse eggs: calcium release in response to sperm or inositol triphosphate is enhanced after meiotic maturation. Biol Reprod 1994; 51:1088–1098.

47. Kubiak JZ, Weber M, de Pennart H, Winston NJ, Mao B. The metaphase II arrest in mouse oocyte is controlled through microtubule-dependent destruction of cyclin B in the presence of CSF. EMBO J 1993; 12:3773–3778.

48. Yanagimachi R. Mammalian fertilization. In: Knobil E, Neil JD (eds.), The Physiology of Reproduction. New York: Raven Press; 1994: 189–317.

49. Bellve A. Purification, culture, fractionation of spermatogenic cells. Methods Enzymol 1993; 225:84–113.

50. Paranko J, Longo F, Potts J, Krohne G, Franke WW. Widespread occurrence of calicin, a basic cytoskeletal protein of sperm cells, in diverse mammalian species. Differentiation 1988; 38:21–27.

51. von Bulow M, Heid H, Hess H, Franke WW. Molecular nature of calicin, a major basic protein of the mammalian sperm head cytoskeleton. Exp Cell Res 1995; 19:407–413.

52. Hess H, Heid H, Franke WW. Molecular characterization of mammalian calicin, a basic protein of the sperm head cytoskeleton. J Cell Biol 1993; 122:1043–1052.

53. Yanagimachi R, Noda YD. Electron microscope studies of sperm incorporation into the golden hamster egg. Am J Anat 1970; 128:429–462.

54. Bedford JM, Calvin HI. The occurrence and possible functional significance of -S-S- crosslinks in sperm heads, with particular reference to eutherian mammals. J Exp Zool 1974; 188:137–156.

55. Swann K, Lai A. A novel signalling mechanism for generating Ca^{2+}-oscillations at fertilization in mammals. Bioessays 1997; 19:371–378.

56. Kuretake S, Kimura Y, Hoshi K, Yanagimachi R. Fertilization and development of mouse oocytes injected with isolated sperm heads. Biol Reprod 1996; 55:789–795.