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Intracytoplasmic Sperm Injection in the Mouse[1]

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ABSTRACT

Intracytoplasmic sperm injection (ICSI) was successful in the mouse when a piezo-driven micropipette was used instead of a mechanically driven conventional pipette. Eighty percent of sperm-injected oocytes survived, and approximately 70% of them developed into blastocysts in vitro. When 106 embryos at the 2- to 4-cell stage were transferred to eight naturally mated foster mothers, 30% of the embryos (25–43%, depending on the host) reached the full term. Except for two that were cannibalized soon after birth, all of the young (30 pups) grew into normal adults. Studies of this type on the mouse may increase understanding of the fertilization process and of how ICSI works.

INTRODUCTION

During normal fertilization, the sperm nucleus is incorporated into an oocyte through membrane fusion between two gametes. Since Uehara and Yanagimachi [1, 2] first reported that sperm nuclei introduced microsurgically into hamster oocytes could transform into well-developed pronuclei, a number of investigators have tried to obtain live young by intracytoplasmic sperm injection (ICSI). While this approach has met with only limited success in animals (rabbits [3] and cattle [4]), it has recently been very successful in humans [5–9]. As of August 1994, the number of ongoing pregnancies from ICSI worldwide was between 500 and 600, with approximately 175 live births having occurred (G.D. Palermo, personal communication).

Since many ICSI experiments cannot be performed in humans for ethical reasons, animal models are desirable. Hamster oocytes are excellent for the study of oocyte activation and pronuclear development after ICSI [10, 11]; however, their use in ICSI studies is hampered because of the difficulty in culturing manipulated oocytes to the transferable stage of development, even though the technique of culturing hamster eggs/embryos has been improving steadily [12]. The mouse is ideal for ICSI experiments because of the ease of culturing eggs/embryos in vitro and the availability of ample genetic information. Unfortunately, ICSI in the mouse has been extremely difficult [13–15]. Years ago, Markert [16] obtained a few mouse blastocysts after ICSI, but all subsequent investigators have failed to confirm this. Here we report that ICSI in mice is possible with the use of a relatively simple technique.

MATERIALS AND METHODS

Reagents

All inorganic and organic reagents were purchased from Sigma Chemical Co. (St. Louis, MO) unless otherwise stated.

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Media

The medium used for culturing oocytes after microsurgery was CZB medium supplemented with 5.55 mM D-glucose [17, 18]. The medium used for collection of oocytes from oviducts, for subsequent treatments, and for micromanipulation was a modified CZB with 20 mM HEPES and reduced $NaHCO_3$ (5 mM).

Preparation of Oocytes

B6D2F1 female mice, 7–11 wk old, were each induced to superovulate by i.p. injection of 7.5 IU eCG followed by 7.5 IU hCG 48 h later. Oocytes were collected from oviducts about 16 h after hCG injection. They were freed from the cumulus by treatment with 0.1% bovine testicular hyaluronidase (300 USP U/mg; ICN Biochemicals, Costa Mesa, CA) in HEPES-CZB for 3–5 min. The oocytes were rinsed thoroughly and kept in CZB for up to 3.5 h at 37°C under 5% CO_2 in air.

Preparation of Spermatozoa

Spermatozoa were obtained from B6D2F1 male mice (8–12 wk old). A cauda epididymis was isolated and placed in HEPES-CZB, and the large tubules were cut in several places to allow the spermatozoa to escape into the medium. Soon after the spermatozoa dispersed into the medium, a drop of concentrated sperm suspension was transferred to 0.5 ml HEPES-CZB and incubated for 5–15 min at 37°C to allow the spermatozoa to disperse evenly in the medium. One part of the sperm suspension was mixed thoroughly with two parts of PVP-saline (0.9% NaCl containing 12% [w/v] polyvinylpyrrolidone [360 kDa; ICN Biochemicals]). A small drop (about 2 μl) of this sperm suspension was kept under mineral oil (Squibb and Sons, Princeton, NJ) in a plastic petri dish on the microscope stage (see below) and kept there for up to 3 h, at 17–18°C, before injection into oocytes. The percentage of motile spermatozoa dropped from nearly 100% in CZB medium to 50–60% upon suspension in the cool PVP-saline. The spermatozoa displayed only

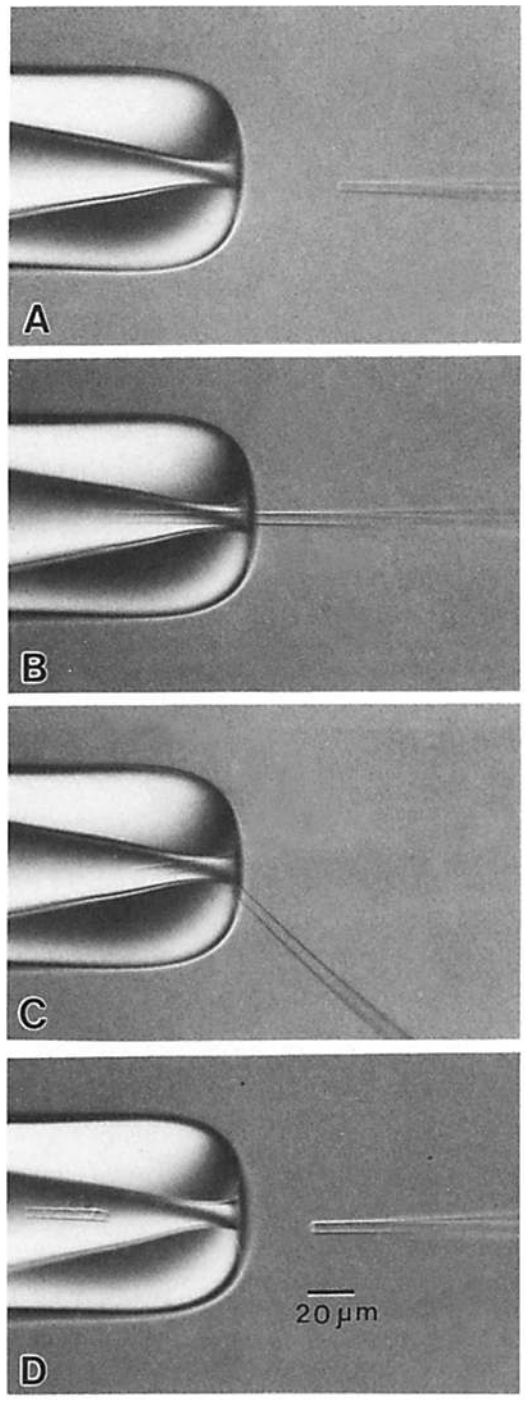


FIG. 1. Preparation of injection pipette for mouse ICSI. A small-bore pipette (A) was inserted into the holding pipette (B) and broken by bending (C, D). If the tip of the pipette was jagged, it was flattened by being tapped gently against the holding pipette.

sluggish movement in the PVP-saline. The percentage of motile spermatozoa dropped to only 5–20% 3 h later.

Preparation of Oocyte-Holding and Injection Pipettes

To prepare holding and injection pipettes, borosilicate glass capillary tubing (1.0 mm o.d., 0.75 mm i.d.; Sutter Instruments, Novato, CA) was drawn with a pipette puller (Model P-97; Sutter Instruments). The oocyte-holding pipette was prepared by scoring the drawn region of the tubing, bending the tube until breakage, and then fire-polishing the pipette end with a microforge (Model MF-79; Narishige Instrument, Tokyo, Japan). The inner diameter of the holding pipette was approximately 10 μm.

Injection pipettes were prepared in two different ways. With the first method (for conventional sperm injection), the tip of the pipette was beveled at a 35° angle with a micropipette beveler (Model BV-10; Sutter Instruments). After the first grind, the pipette was rotated (90°) and then given an additional grind to make a sharply pointed tip. The inner diameter at and near the pipette tip was approximately 5 μm. A small volume (about 0.5 μl) of mercury was placed in the proximal end of the injection pipette, which was then connected to a water-filled syringe system in a Leitz micromanipulator. After the mercury was pushed to the tip of the pipette, a small volume of mineral oil (5–10 pl) was sucked into the pipette. With the second method (for sperm injection using the piezo-microinjector; see below), the pipette tip was immersed in a 25% solution of hydrofluoric acid (Baker Chemical Co., Phillipsburg, NJ) while the solution was drawn in and blown out of the pipette several times [19]. The pipette was thoroughly rinsed with distilled water, dipped in 100% ethanol, and dried. Mercury and mineral oil were placed in the pipette as described above. Immediately before sperm injection, the tip of the pipette was broken such that the inner diameter at and near the tip was 4.5–5.0 μm (Fig. 1). If the tip of the pipette was jagged, it was flattened by being gently tapped against the holding pipette.

Sperm Injection into Oocytes

The cover (10 mm in depth) of a plastic dish (100 mm × 15 mm; Falcon Plastics, Oxnard, CA, cat. no. 1001) was used as a microinjection chamber. A row consisting of two round droplets and one elongated drop was placed along the center line of the dish. The first droplet (2 μl; 2 mm in diameter) was for pipette washing (PVP-saline). The second droplet (2 μl; 2 mm in diameter) was the sperm suspension in PVP-saline. The third elongated droplet (6 μl; 2 mm wide and 6 mm long) was HEPES-CZB medium for the oocytes. These droplets were covered with mineral oil (Squibb and Sons). The dish was placed on the cooled (17–18°C) stage of an inverted microscope (Leitz Labovert FS, Foster City, CA) with Nomarski differential interference optics. Our preliminary experiments revealed that postoperation survival of the oocytes was much better at this temperature than at higher temperatures (25–37°C).

Sperm injection was carried out in two different ways.

Method 1. One of the methods used was the conventional one [11, 20]. Briefly, a single spermatozoon moving slowly in the PVP-saline was picked up, tail first, into the injection pipette. Sucking in and blowing out of the pipette

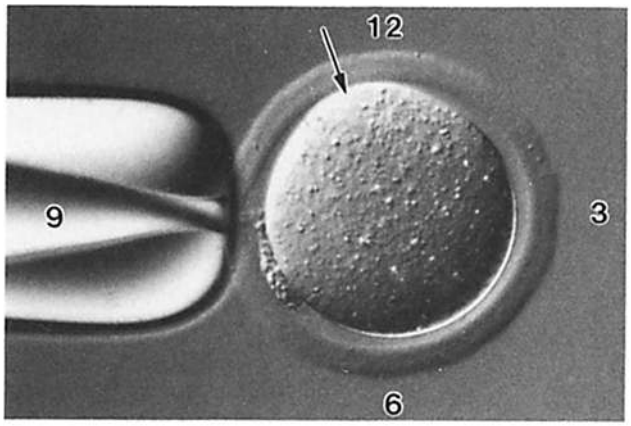


FIG. 2. Ideal positioning of the oocyte prior to ICSI. An oocyte on the holding pipette was rotated until the metaphase II spindle, indicated by a "hump" (arrow) in the cortex, was around the 12 o'clock position. Injection pipette is to be inserted from the 3 o'clock direction.

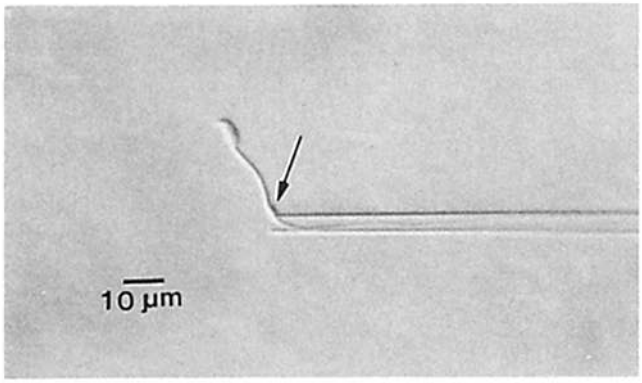


FIG. 3. Immobilization of a spermatozoon prior to ICSI. A motile spermatozoon was sucked, tail first, into the injection pipette. When the midpiece-endpiece junction (arrow) was in the opening of the pipette, two to three piezo-pulses were given to immobilize the spermatozoon instantly.

was repeated until the spermatozoon was immobilized. This immobilized spermatozoon was sucked deeply into the pipette tail first. An oocyte was held to the holding pipette at the 9 o'clock position and then rotated until the metaphase II spindle (as indicated by a hump in the oocyte's cortex) was around either the 12 or the 6 o'clock position (Fig. 2). There were two reasons for the positioning used. First, such positioning would avoid damage to the metaphase II spindle that could be caused by deep insertion of a pipette into the oocyte from the 3 o'clock position. Second, the oocyte cortex in the vicinity of the metaphase spindle is very tough, most probably because of the presence of abundant actin filaments [21]. Damaging this region appeared to disturb normal extrusion of the second polar body. At the 3 o'clock position the zona was pierced through by a sharply pointed injection pipette containing a spermatozoon. After the tip of the pipette had been inserted approximately one third of the way to halfway through the oocyte, the pipette was pushed forward swiftly at the same time as the oocyte's cortex was drawn into the pipette. This motion broke the oolemma. The spermatozoon was then expelled into the ooplasm with a minimum amount (about 6 pl) of accompanying medium. One to two seconds later, the sperm-suspending medium, which was still visible around the tip of pipette, was retrieved to the extent possible, with great care being taken not to suck an excessive amount of the ooplasm. The pipette was withdrawn gently. On average, it took 10–20 min to inject spermatozoa into a group of 5–10 oocytes.

Method 2. This is a new method using a piezomicropipette-driving unit (sold under the name of Piezo Micromanipulator Model PMM-01 by Prima Meat Packers, Tsuchiura-City, Ibaraki-ken, Japan). This unit, based on the piezoelectric effect, is able to advance the pipette holder a very short distance (e.g., 0.5 µm) at a time at a very high speed. A stabbing, punctate movement of an attached pipette punctures the cell membrane (oolemma) with minimum distortion of the cell (oocyte). We noted that the presence of a mercury droplet near the tip of the injection pipette increased the penetrating capability of the pipette through the zona pellucida and oolemma. We also noticed that more sperm-suspending medium and a smaller amount of oil within the pipette tip increased the piercing ability of the piezo-driven pipette. The reasons for these facts are not clear at present.

A motile spermatozoon was sucked, tail first, into the injection pipette. When the junction between the midpiece and the principal piece (where the cytoplasmic droplet was commonly observed) was in the opening of the pipette (Fig. 3), two to three piezo-pulses were applied to immobilize the spermatozoon. The intensity and speed (= frequency) of the pulse were regulated by the controller PMAS-CT01 (controller setting scales: intensity 2, speed 1). The entire length of the spermatozoon was then drawn deep into the pipette (see below). An oocyte was positioned as described above (Fig. 2). After the tip of the injection pipette was brought into intimate contact with the zona at the 3 o'clock position (Fig. 4A), several piezo-pulses (intensity 1–2, speed 1–2) were given to advance the pipette while a light negative pressure was applied to it. When the tip of the pipette had passed through the zona, a cylindrical piece of the zona in the pipette was expelled into the perivitelline space (Fig. 4B). After the spermatozoon was pushed forward until its head was near the tip of the pipette (Fig. 5A), the pipette was advanced quickly through the ooplasm until its tip almost reached the opposite side of the oocyte's cortex (Fig. 5B). This was done mechanically. It was rather surprising that the oolemma could stretch that much without being broken. Upon application of one to two piezo-pulses (intensity, 1–2, speed 1), the oolemma was punctured at the pipette tip, as indicated by a rapid relaxation of the oolemma (compare Fig. 9b with 9a). The spermatozoon was then expelled into the ooplasm with a minimum amount (about 6 pl) of accompanying sperm suspension medium

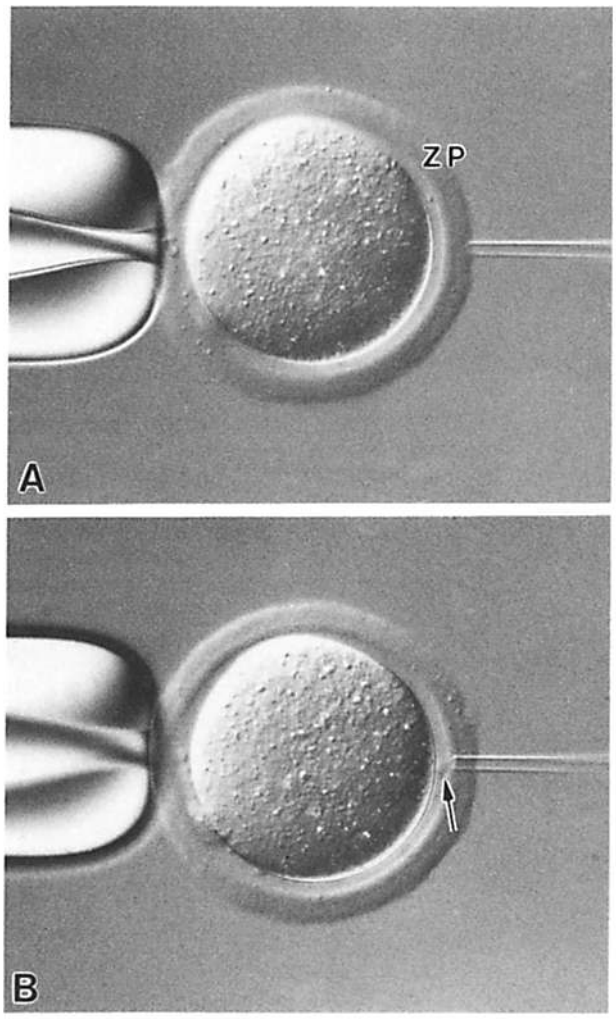


FIG. 4. Zona-drilling prior to ICSI. **A)** The tip of the injection pipette was brought into contact with the zona pellucida (zp). **B)** After the piezo-driven pipette had passed through the zona, a piece of the zona in the pipette was expelled into the perivitelline space (arrow).

(Fig. 5, C-D). After as much as possible of the medium was retrieved (Fig. 5E), the pipette was gently withdrawn, leaving the spermatozoon within the ooplasm (Fig. 5F). We routinely applied a light negative pressure to the pipette during its withdrawal. This appeared to increase the survival rate of the oocytes. A group of 5–20 oocytes were each microinjected with a single spermatozoon within 10–15 min. Oocytes undergoing this operation in exactly the same manner, but without the introduction of a spermatozoon, served as sham-operated controls.

Incubation and Examination of the Ova

Sperm-injected oocytes were kept in the operation medium (HEPES-CZB) for about 20 min on the cooled (17–18°C) stage of the microscope. They were then transferred into a dish of HEPES-CZB medium at room temperature (24–25°C). Ten minutes later, the oocytes were transferred into 50 μl CZB medium under mineral oil in a plastic dish (35 × 10 mm; Falcon Plastics, cat. no. 3001) and incubated at 37°C under 5% CO_2 in air. Some were fixed for electron microscopy between 0.5 and 2 h after the start of incubation at 37°C. Others were incubated at 37°C for 5–7 h and examined under an inverted microscope with a 10× objective. Eggs with distinct signs of degeneration were counted and discarded. Those with two distinct pronuclei and the second polar body were considered fertilized and were cultured continuously. Others (e.g., those having no pronuclei or those having a single pronucleus or three pronuclei) were fixed and stained for observation of cytological details [11]. Embryos developing in vitro were examined with the inverted microscope at 24-h intervals for up to 120 h after sperm injection.

Embryo Transfer into Foster Mothers

Recipients of the embryos were Swiss-Webster (albino) mice maintained in a light-controlled room (14L:10D, lights-on at 0500 h). They were mated naturally with males of the same strain. The ages of females and males at the time of mating were 7–9 wk and about 20 wk after birth, respectively. The day on which a vaginal plug was found was defined as Day 1 of pregnancy. At about 1400 h on Day 1, females were anesthetized with sodium pentobarbital, and their oviducts were exposed through dorso-ventral incisions. Meanwhile, the embryos (2- or 4-cell stage) to be transferred were sucked into an embryo transfer pipette (100–150 μm in diameter at its tip). The oviductal ampulla was pierced with a sharply pointed 25-gauge needle slightly above the distended region of the ampulla. This allowed a blunt-end embryo transfer pipette to be inserted easily into the lumen of the ampulla. The number of embryos transferred into each oviduct was 5–7.

The zonae pellucidae of about half of the embryos were partially dissected to determine whether or not zona dissection facilitates the development (hatching/implantation) of embryos. Each embryo was sucked with the holding pipette (at the 9 o'clock position) such that a large perivitelline space appeared at the opposite side. A piezo-driven injection pipette with a flat tip (i.d., about 8 μm) was applied repeatedly against the zona until a large hole (about 20 × 10 μm in diameter) was produced in the zona at the 3 o'clock position. Care was taken to avoid direct contact of the pipette with the embryo proper. Zona dissection was carried out on the warm (about 37°C) stage of the microscope.

Foster mothers were allowed to deliver and raise their own young (red eyes and white coat) as well as foster pups (black eyes and grey/brown/black coats).

RESULTS

Sperm injection via a piezo-driven micropipette was far less traumatic to the oocytes than the conventional method

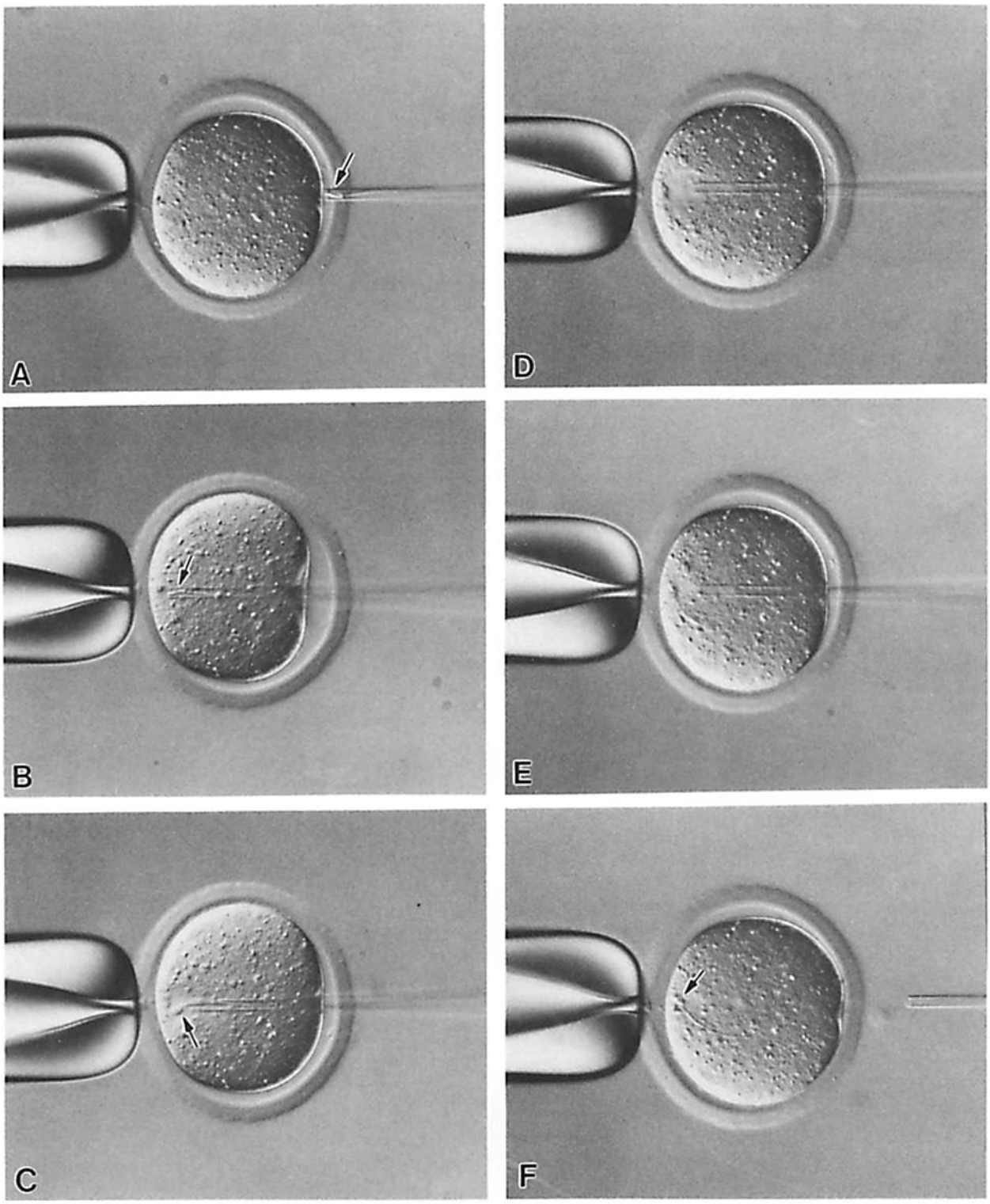


FIG. 5. ICSI using piezo-driven pipette. Sperm injection pipette was inserted deeply into an oocyte (**A, B**). After the oolemma was broken by application of piezo-pulses, the entire spermatozoon was injected (**C, D**). Medium injected with the spermatozoon was retrieved as much as possible (**E**) before withdrawal of the pipette (**F**). Arrow indicates sperm head. For details, see the text.

TABLE 1. Response of mouse oocytes to sperm injection and sham operation, examined between 5 and 7 h after injection.

Sperm-injection method	No. of experiments	Total no. oocytes injected	No. (%) oocytes survived*	No. (%) eggs with			
				Met II	2 PN + Pb_2	1 PN + Pb_2	3 PN
Conventional	6	112	18 (16)[a]	7 (6)	9 (8)	1 (1)	1 (1)
Piezo-driven	11	322	256 (80)[b]	0	251 (78)	0	5 (2)
Piezo-driven (Sham control)	6	65	63 (97)[c]	62 (95)	0	1 (2)	0

*Differences are significant between a and b ($p < 0.0001$) and between b and c ($p < 0.0007$), Fisher's Exact test.

using a mechanically driven pipette. While only 16% of the oocytes survived after sperm injection by the conventional method, the majority (80%) survived with the new method using a piezo-driven micropipette (Table 1). The incidence of normal fertilization, as evidenced by the presence of two large pronuclei and a distinct second polar body, was about 10 times higher in piezo-operated eggs than in the conventionally operated eggs. It should be noted that the vast majority of the sham-operated oocytes remained unactivated; only 2% were activated parthenogenetically (Table 1).

The eggs fertilized by sperm injection could develop in vitro regardless of the sperm injection method, but those operated on with a piezo-driven pipette developed better than those on which the conventional method was used (Table 2).

Figure 6 illustrates mouse oocytes/eggs at various times after ICSI. Sperm nuclei, which were "intact" immediately after injection (Fig. 6, A and B), were decondensed by 1.5 h after injection. Electron micrographs of spermatozoa at this stage and at earlier stages are shown in Figure 7. At 1 h after sperm injection, plasma membranes in the head regions were largely missing; the sperm nucleus was decondensing (Fig. 7A) and cortical granules were absent in the egg cortex (not shown). By 1.5 h after injection, the nucleus was fully decondensed (Fig. 7B); the acrosome membrane was partially broken and the acrosomal contents were in direct contact with the ooplasm (Fig. 7C).

The majority of the oocytes injected with spermatozoa via a piezo-driven pipette developed normally in vitro (Fig. 6, D-F). All eight foster mothers receiving sperm-injected eggs delivered their own and foster offspring (Table 3; Fig. 8). Zona-drilling prior to embryo transfer had no obvious effect on development/implantation of sperm-injected eggs. All 30 foster pups (not including two that were cannibalized within a few days after birth) grew into normal adults. The sex ratio of foster offspring was 1:1. Six males and six females that had been randomly selected were mated, and all proved to be completely fertile.

DISCUSSION

Why Has Mouse ICSI Been Difficult in the Past?

Our preliminary experiments revealed that the "wound-healing" capacity of mouse oocytes is inferior to that of hamster and human oocytes. When the mouse oolemma was punctured via the piezo-driven pipette without the pipette's being deeply inserted, the oocyte's contents (ooplasm) oozed out from the point of puncture to disperse rapidly into the surrounding medium. All the oocytes degenerated sooner or later. In similarly treated hamster and human oocytes, the ooplasm at the point of puncture remained in situ, and most oocytes survived. Apparently the coherence (viscosity) of the ooplasm as well as the wound-healing ability of the oolemma is much higher in the hamster and human than in the mouse. We also noticed that the oolemma of mouse oocytes was far more elastic than that of hamster and human oocytes. When a conventional sperm injection pipette was pushed against a hamster oocyte, the zona was indented, and then both the zona and oolemma were broken (pierced by the pipette) abruptly. This occurred before the tip of the pipette reached near the center of the oocyte. In the mouse, the zona could be broken by a sharply pointed pipette, but the oolemma did not break even when the pipette tip almost reached the cortex of the opposite side of the oocyte. When a spermatozoon in the pipette was expelled at this position, the spermatozoon appeared as if it had been successfully deposited within the ooplasm. In reality, however, it was still outside the ooplasm. It was merely trapped in the oocyte,

TABLE 2. Developmental ability of mouse eggs fertilized by sperm injection.

Sperm injection method	No. of experiments	Total no. of cultured eggs	No. (%) eggs developed into			
			2-cell	4-cell	Morula	Blastocyst
Conventional	3	9	9 (100)	9 (100)	7 (78)	3 (33)*
Piezo-driven	6	119	118 (99)	115 (97)	108 (91)	81 (68)*

*Difference is not quite significant ($p = 0.0624$, Fisher's Exact test) due to the low numbers of the control group.

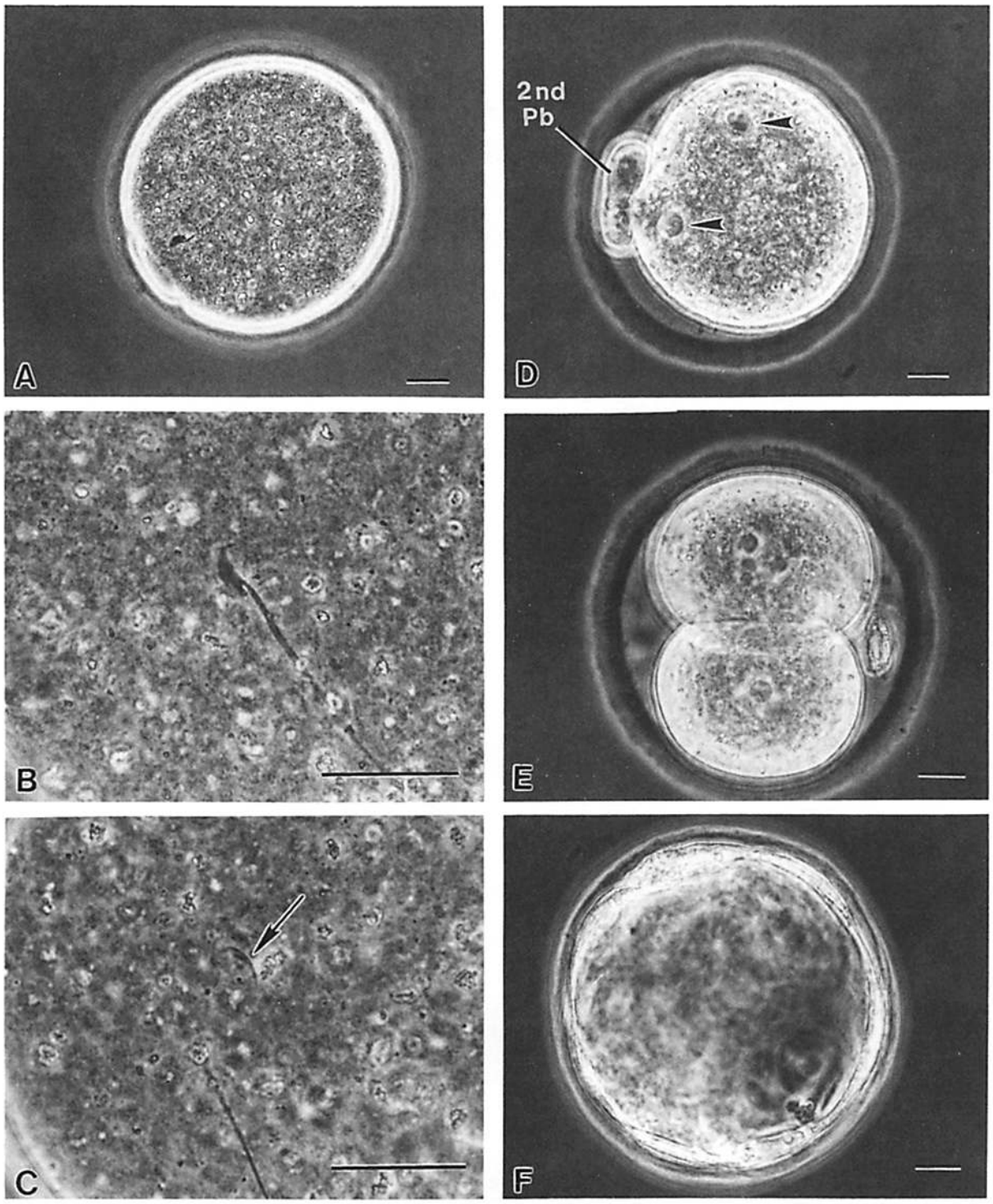


FIG. 6. Phase-contrast micrographs of living eggs after ICSI. Eggs were more or less compressed between a slide and coverslip before photography. Bar equals 20 μm. **A)** An oocyte immediately after sperm injection. **B)** The same, but under higher magnification. **C)** A swollen sperm head in an egg, 1 h and 45 min after injection; arrow indicates a remnant of the acrosomal cap and/or subacrosomal material. **D)** A pronuclear egg, 5 h after sperm injection; arrowheads indicate sperm and egg pronuclei; 2nd Pb, second polar body. **E)** A 2-cell egg, 1 day after injection. **F)** A blastocyst, 4 days after injection.

surrounded by the medium and the stretched oolemma. Among 18 oocytes that survived after sperm injection via the conventional pipette, seven were unactivated (still arrested at metaphase II) and one was activated but had only one pronucleus (cf. Table 1). Each of these oocytes had an intact sperm head either within or on its surface. We sus-

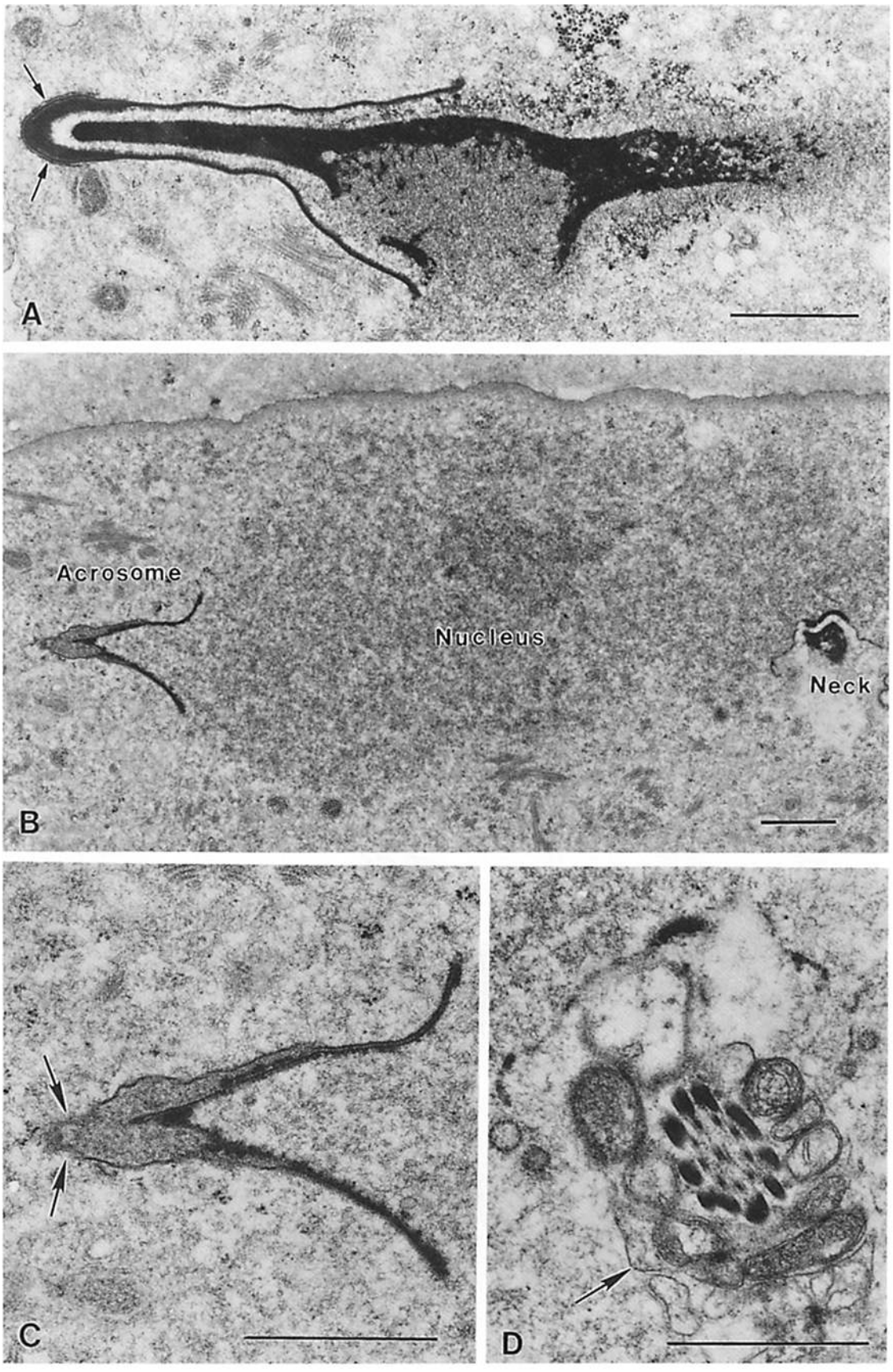


TABLE 3. The results of embryo transfer

Recipients	Zona-drilled Prior to transfer	No. of embryos transferred (dev. stage)	No. of live offspring[a]		
			Own (albino)	Foster (black/gray)	Sex of foster offspring[b]
a	Yes	12 (2-cell)	7	3	1M, 2M
b	Yes	14 (2-cell)	0	4	2M, 2F
c	Yes	14 (4-cell)	5	5	1M, 3F, 1?
d	Yes	14 (4-cell)	3	6	3M, 3F
e	No	12 (2-cell)	3	3	2M, 1F
f	No	14 (2-cell)	4	4	2M, 2F
g	No	14 (4-cell)	5	4	2M, 2F
h	No	12 (4-cell)	10	3	2M, 1?
Total		106	37	32	15m, 15F, 2?

[a]The origin of offspring was identified by eye color (on the day of birth or 1–2 days after birth) and fur color (later date).
[b]Babies were sexed between 1 and 4 wk after birth. Two pups were canibalized by mother soon after birth, so their sex remained undetermined.

pect that all of these oocytes had either unbroken or incompletely broken oolemmae. The spermatozoa we saw outside of unactivated oocytes must have been pushed out of the oocytes sometime after withdrawal of the pipette. It was, of course, possible to break the mouse oolemma with the conventional pipette. From time to time, perhaps depending on the size and shape of the pipette tip, the pipette pierced the oolemma rather easily and oocytes survived after sperm injection and pipette withdrawal. In most cases, however, repeated stabbings were necessary to break the oolemma, and this seemed to damage the oolemma irreversibly. Almost all of the stabbed mouse oocytes degenerated after withdrawal of the pipette, indicating that the extensively damaged oolemma was not able to heal.

Why Does the Method Using the Piezo-Driven Pipette Work Better Than the Conventional Method?

As shown in Table 1, ICSI using a piezo-driven micropipette was far more effective than that using the conventional pipette. In contrast to the conventional pipette, the piezo-driven pipette could break the oolemma readily and consistently. When the oolemma at the tip of a deeply inserted pipette was broken by piezo-pulses, the extended oolemma began to revert or relax (Fig. 9, A and B). As the pipette was withdrawn after sperm deposition, the oolemma further reverted (Fig. 9C) until it completely recovered its original shape. At high temperatures (e.g., 25–37°C), this reversion occurred quickly, and many oocytes degenerated. Perhaps the oolemma reverted before the broken oolemma "healed" completely, resulting in an influx of the surrounding medium into the oocyte (Fig. 9,

FIG. 7. **A)** Sperm nucleus in an egg kept at low temperature for 30 min after ICSI and then incubated at 37°C for 30 min. Nuclear decondensation was in progress; arrows indicate disintegrating plasma membrane. **B)** Fully decondensed sperm nucleus in an ICSI-fertilized egg that had been kept for 30 min at low temperature and then for 1 h at 37°C. **C)** The acrosome of the same spermatozoon under higher magnification; sperm plasma membrane over the acrosome and part of the outer acrosomal membrane has disappeared, and acrosomal contents are in direct contact with the ooplasm (arrows). **D)** Midpiece near the neck region of the same spermatozoon; the membrane indicated by the arrow is probably the disintegrating sperm plasma membrane. Each bar equals 1 μm.

FIG. 8. A female mouse with her own four white pups and four colored foster pups (arrows). The latter developed from sperm-injected oocytes.

D-F). At lower temperatures (17–18°C), the reversion process was slowed down, perhaps because of an increase in the viscosity (coherence) of the ooplasm. This slow reversion seems to keep the oolemma of the pipette-made channel closed for an extended period of time, preventing influx of the medium into the oocyte. It must be that the broken oolemma is healed during this period (Fig. 9, G-I). A key to success in ICSI was avoiding leakage of the medium from the pipette during its withdrawal. The medium left by the pipette appeared to disturb the closure of the pipette-made channel, thus allowing an influx of the surrounding medium into the oocyte. We avoided this by applying light negative pressure to the pipette during its withdrawal.

Is Sperm Immobilization Prior to ICSI Necessary?

In the present study we immobilized spermatozoa immediately before injection into oocytes. Repeated sucking in and out of the injection pipette or application of piezo-pulses must have damaged the sperm plasma membrane. Immobilization of spermatozoa, however, was not essential for successful ICSI (Y. Kimura and R. Yanagimachi, unpublished data). When motile spermatozoa were injected, they kept moving within the ooplasm for a few minutes, sometimes for as long as 20 min, before they became motionless. Although some oocytes operated on in this way reached full term (Y. Kimura and R. Yanagimachi, unpublished data), we temporarily abandoned this approach because continuous movement of spermatozoa within the ooplasm may disorganize the oocyte's structural elements and/or disturb the oolemma's "wound healing." However, whether or not sperm immobilization prior to ICSI does really increase the success rate is an open question.

Oocyte Activation after ICSI

It is interesting to note that a simple breaking of the mouse oolemma by the piezo-driven pipette (sham control) seldom activated the oocytes (Table 1). During sham operation with a piezo-driven pipette, a small amount of the medium (PVP-saline) was injected into the oocyte. Thus it must have been the spermatozoon itself, not the medium, that activated the oocyte. Introduction of an oocyte-activating factor (or protein?) by spermatozoa during normal fertilization has been postulated [22–26], and the results of the present study seem to support this view. We are currently investigating the nature of this factor in spermatozoa.

Differences between ICSI-Fertilized and Normally Fertilized Eggs

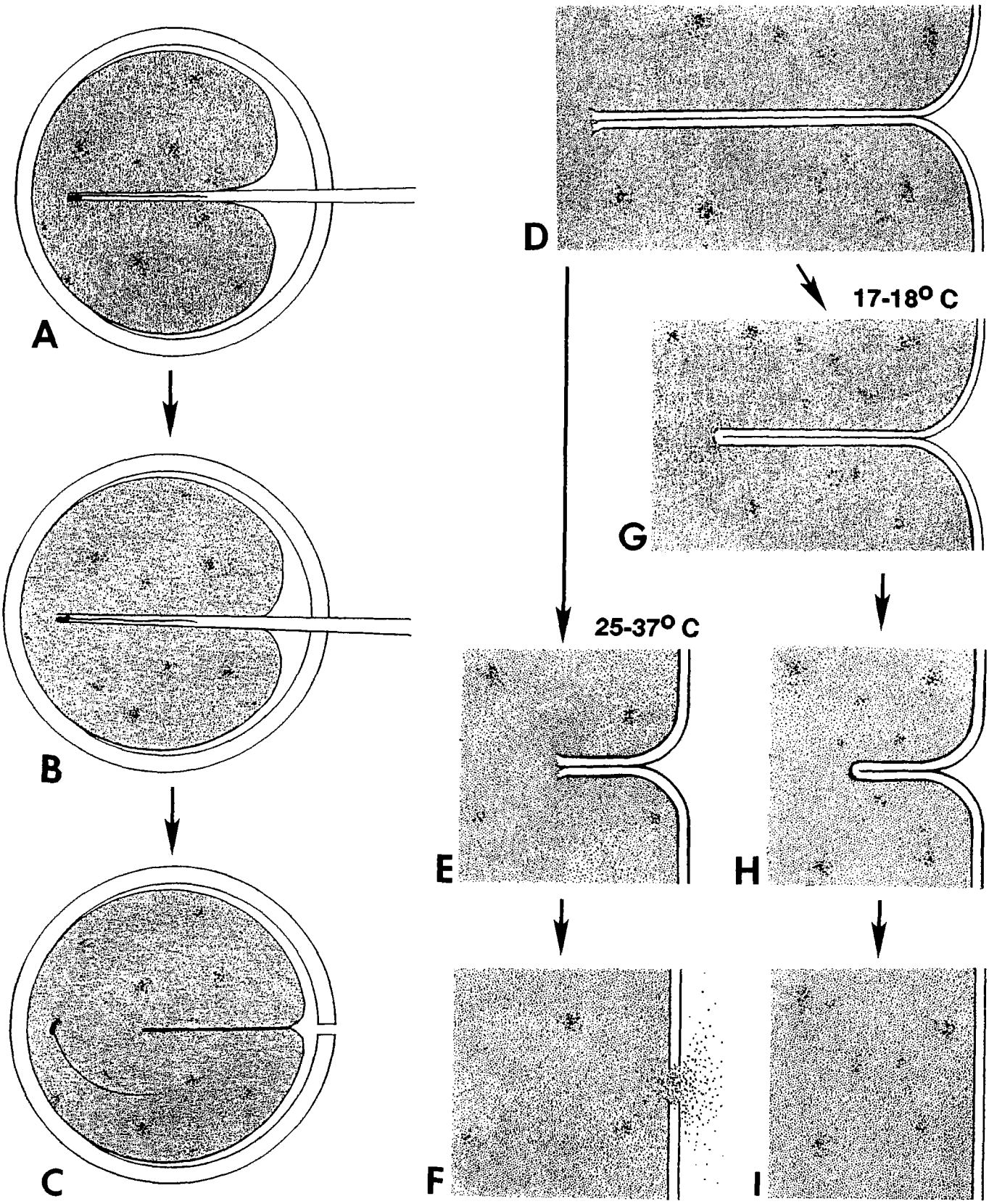
In normal fertilization, either in vivo or in vitro, sperm plasma membrane mingles with the oocyte's plasma membrane to become the oolemma of the zygote. Acrosomal contents, rich in hydrolyzing enzymes, are released during the acrosome reaction and never enter the oocyte [27]. During ICSI the entire spermatozoon, including the whole plasma membrane and acrosome, was injected into an oocyte. Despite this unusual situation (and an initial 30-min exposure of sperm-injected eggs to low temperature), the speed of sperm nucleus decondensation in ICSI-fertilized eggs was about the same as in normally fertilized eggs. The onset of the first and subsequent cleavages was also about the same in ICSI-fertilized and normally fertilized eggs.

When ICSI-fertilized eggs were examined by electron microscopy within a few hours after sperm injection, the acrosomes were partially broken (Fig. 7). Although we did not follow the fate of the acrosome after ICSI, it must be eliminated in one way or another sometime during embryonic development. We found that mouse oocytes injected with acrosome-intact hamster or rabbit spermatozoa deformed grossly in several hours, whereas those injected with spermatozoa from which acrosomes had been removed did not (Y. Kimura and R. Yanagimachi, unpublished data). Since the acrosomal cap of hamster and rabbit spermatozoa is much larger than that of the mouse spermatozoon [27], this observation seems to indicate that the mouse oocyte has limited ability to handle (eliminate) exogenous acrosomal enzymes. For species in which the sperm's acrosome is large and the oocyte is relatively small, it may be necessary to remove acrosomes from spermatozoa prior to ICSI. For ICSI in humans, relatively large oocytes and small spermatozoa (small acrosomes) probably make removal of the acrosomes unnecessary.

Embryos developed from sperm-injected oocytes appear to be as competitive as native embryos for implantation sites (Table 3). Zona-drilling, which we thought beneficial for hatching/implantation of the embryos, did not affect the outcome of embryo transfer.

There is still room for improvement of mouse ICSI. The oocyte survival rate, which is currently about 80% (Table 1), should be improved further. In the present study we used hybrid mice. Oocytes of inbred strains may or may not be as tolerant to ICSI as hybrid mice. This must be studied further. When the technique is perfected, the mouse would be a good animal model for ICSI in view of the availability of ample genetic information in this species. Mouse ICSI will be valuable for designing and improving human and animal ICSI when experiments are difficult for ethical and various other reasons.

FIG. 9. Response of mouse oocyte to piezo-driven pipette (**A-C**) and hypothetical view of the healing process of the oolemma (**D-I**). A micropipette front-loaded with a spermatozoon is inserted deep into the oocyte (**A**). When the oolemma is broken by a piezo-driven pipette, stretched oolemma relaxes (**B**). As the pipette is withdrawn after sperm injection, the pipette-made channel closes (**C**) and becomes shorter with time. At high temperatures (25–37°), oolemma reverts quickly before the oolemma heals (**D,E**), resulting in cytolysis of the oocyte (**F**). At low temperatures (17–18°C), the reversion of the oolemma is slowed down, and the oolemma heals before it reverts completely (**G-I**).



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