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INTRACYTOPLASMIC SPERM INJECTION (ICSI) IN MICE BY A NEW HOLDING PIPETTE

AMIR ESMAILNEJAD MOGHADDAM, Ph.D., ABBASALI KARIMPOUR MALEKSHAH, Ph.D., MASAHIKO HIROI,* M.D. AND HIDEKAZU SAITO,* M.D.

*From the Department of Anatomy and Embryology, Medical School, Mazandaran University of Medical Sciences, Sari, I.R. Iran, and the *Department of Obstetrics and Gynecology, Medical School, Yamagata University, Yamagata, Japan.*

ABSTRACT

Mice are ideal models for ICSI experiments because of the ease of culturing eggs/embryos *in vitro* and the availability of ample genetic information. Unfortunately, it has been extremely difficult. In this study we improved the mouse ICSI method by using a new holding pipette that was made of two pipettes such that one pipette was pulled and heat merged into the other one. The outer pipette had an outer diameter of 120μ and an inner diameter of 80μ to 85μ (about mouse oocyte diameter).

The inner pipette had an outer diameter of 80μ to 85μ and an inner diameter of 55μ to 60μ which was polished and narrowed to 20μ on a microforge. The distance between the tips of the two pipettes was adjusted to 120μ to 160μ (1.5 to 2 fold longer than mouse oocyte diameter). Of 307 oocytes which were injected with a single spermatozoon, 206 (67.1%) survived and 93 (45.1%) of surviving oocytes showed normal fertilization (2 pronuclei and second polar body). Of 109 oocytes which were only sucked into the holding pipette (control group), 105 (96.3%) survived and only 4 (3.8%) of them became activated parthenogenically.

Using this new holding pipette, the oocyte is sucked into a glass tunnel and elongates to a reasonable length; therefore the injection axis will be increased and piercing of the oolemma can be performed easily.

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INTRODUCTION

The intracytoplasmic sperm injection of human spermatozoa into human eggs (ICSI) now seems to be the most beneficial approach to obtain fertilization to give births in cases of male infertility.[1-3] However, many questions are still left unanswered,[4] so different aspects of this new method, especially the biological mechanisms of it, should be understood more.[5,6]

Many ICSI experiments cannot be performed in humans for ethical reasons, hence animal models are desirable. Although the hamster is an excellent animal model for the

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Correspondence: A. Esmailnejad Moghaddam, Ph.D., Dept. of Anatomy and Embryology, Medical School, Mazandaran University of Medical Sciences, Sari, I.R. Iran.

studies of some aspects of ICSI, culture and subsequent transfer of their zygotes to foster mothers are still very difficult, if not impossible.[7]

The mouse is ideal for ICSI experiments because of the ease of culturing eggs/embryos *in vitro* and the availability of ample genetic information.[8] Unfortunately ICSI in the mouse has been extremely difficult. There are some main differences with mouse ICSI.[9] The diameter of the mouse oocyte (80μ) is almost one-half the size of the human oocyte (150μ). The mouse spermatozoon has a three-fold longer tail (140μ) than the human spermatozoon (50μ), thus the volume of PVP/medium injected into the ooplasm is approximately three-fold higher than with human oocytes. The mouse spermatozoon has a hooked acrosome which causes the spermatozoon to adhere to the inner wall of the pipette, thus it is necessary to use a pipette of 7-8μ in diameter. Also the perivitelline membrane of the mouse oocyte has a high elasticity which causes difficulty in penetrating the oocytes.[10]

In this study, we improved the mouse ICSI method by making and using a new holding pipette.

MATERIALS AND METHODS

Media

The medium used for collection of oocytes from oviducts, sperm suspension and micromanipulation was HEPES-buferred human tubal fluid (HTF) medium.

Preparation of oocytes

Mouse oocytes were obtained from B6C3F1, 5-10 weeks old, following stimulation with 10 IU pregnant mare's serum gonadotropin (PMSG; Folligon, Intervet, Lane cove 2066, Australia). Ovulation was induced with 10 IU human chorionic gonadotropin (HCG; Pregnyl, Organon, Profasi, Serono) 48 hours later. Animals were killed by cervical dislocation, 13-14 hours after HCG administration. The cumulus was removed by treatment with 1 mg/mL hyaluronidase (Sigma Chemical Co., St. Louis, MO, USA) for 2-3 minutes. The oocytes were washed and transferred in HTF medium supplemented with 50 mg/mL human albuminar serum (Sigma Chemical Co.), and then they were incubated in a humidified atmosphere of 5% CO_2 in air at 37° C. Oocytes were cultured for at least 2 hours before injection. Those oocytes which extruded a second polar body during this time were considered to be activated by the hyaluronidase treatment and were discarded. Only oocytes with a small perivitelline space, a homogenously granulated cytoplasm and remnants of the first polar body were used for the microinjection.

Preparation of spermatozoa

Sperm suspsension was prepared by releasing the cauda epididymis from mature B6C3F1, 8-12 week old mice in 1 mL HEPES-buferred HTF, and was preincubated for a total of 120 minutes for capacitation. A small drop of this suspension (debris-free) was kept under mineral oil in a Nunc tissue culture dish (Nunc Inter Med, Roskilde, Denmark).

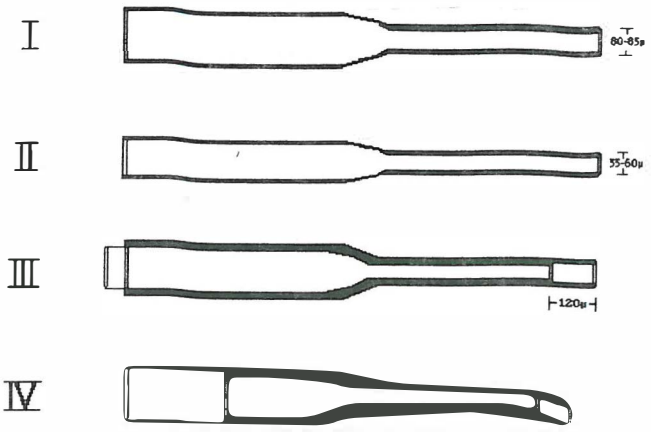

Fig. 1. Construction of the new holding pipette.

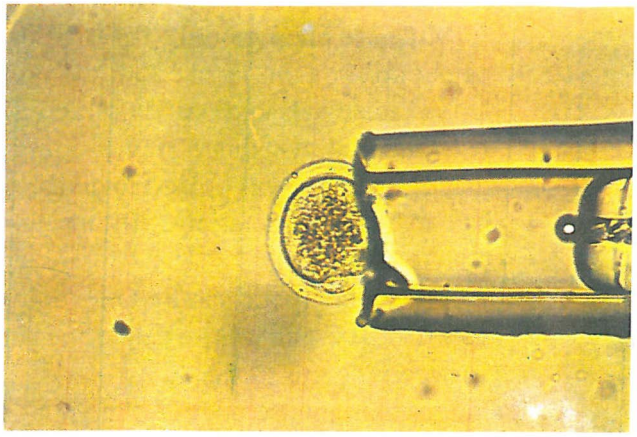

Fig. 2. The oocyte is sucked gently into the holding pipette with the polar body at 12 or 6 o'clock position.

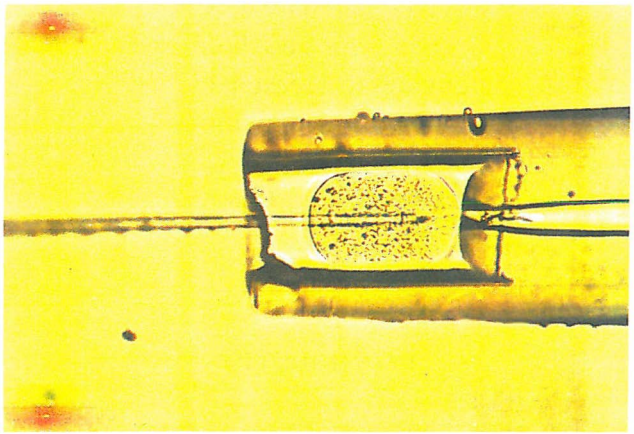

Fig. 3. The injection pipette is inserted through the zona pellucida.

Table I. Results of intracytoplasmic sperm injection in mouse oocytes.

Groups	No. of experiments	Total oocytes	Survived oocytes (%)	Met II (%)	2pn, pb2 (%)	1pn, pb2 (%)	3pn (%)	Activated oocytes (%)
ICSI	13	307	206(67.1)*	105(51)	93(45.1)	5(2.4)	3(1.5)	8(3.9)
Control	13	109	105(96.3)*	101(96.1)	0	4(3.8)	0	4.(3.8)

*significantly different ($p<0.0001$).

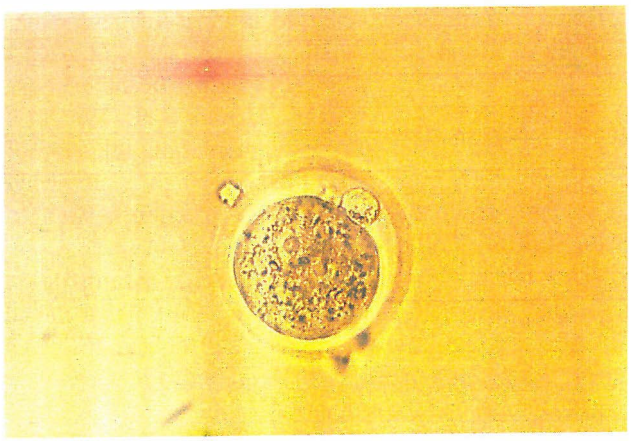

Fig. 4. The fertilized oocyte with two clear pronuclei and a second polar body.

Microtools for ICSI

Injection pipettes were made from glass capillaries (outer and inner diameters of 1 mm and 0.75mm, length 100 mm, Suter Instruments Co., Novato, CA) using a puller, microgrinder and microforge MF9 from Narishige (Narishige Co. Ltd, Tokyo, Japan). This needle was prepared by two stage pull of a capillary tube on a micropipette vertical puller to an outer diameter of 8-9μ and inner diameter of 7-8μ. The tip of the microneedle was ground backed to a bevel angle of 45° with the microgrinder. Using a microforge, a sharp spike was pulled on the tip of the microneedle and then the edge of it was bent to an angle of 30° to facilitate the injection procedure in the petri dish.

Holding pipettes were made from G-1 Narishige glass capillaries (outer and inner diameter of 1mm and 0.80 mm, Narishige Co. Ltd, Tokyo, Japan). Our new holding pipette was made in three steps (Fig.1): (i) A glass pipette was prepared with pull of the capillary tube on the horizontal puller and the tip of it was cut to obtain an outer diameter of 120μ and an inner diameter of 80-85μ (about mouse oocyte's diameter). Another glass pipette was prepared by hand-pulling the capillary on the fire and cutting the tip to obtain an outer and inner diameter of 80-85μ and 55-60μ, respectively. The end of the latter one was polished and narrowed to 20μ on a microforge. (ii) The latter pipette was taken in the first one through the wide opened end of it and was fixed temporarily using a piece of paste; so that the distance between the tips of the two pipettes was adjusted to 120-160μ (1.5 to 2 fold longer than mouse oocyte diameter). (iii) The external wall of the inner pipette was merged to the internal wall of the outer pipette by gently heating at the side about 400-500μ from the tip of the outer pipette, using the microforge; concomitantly the edge of the micropipette would be bent. The small distance between the inner diameter of the outer pipette and the outer diameter of the inner pipette, and also proper heat adjustment of the microforge are very important for preparing a good pipette. After merging, the inner pipette was cut above the merging point by pulling it gently. The pipettes were washed by HF and distilled water to remove particles. It is worth mentioning that preparing a suitable internal diameter for the holding pipette is very important; which should be about the size of the mouse oocyte (indeed a little smaller than it). If the internal diameter of the holding pipette is too small compared to the oocyte's diameter it will cause high pressure upon the oocyte and therefore oozing of the cytoplasm will occur after withdrawal of the injection needle.

Micromanipulation

Micromanipulation was performed under an Olympus inverted microscope (Olympus Optical Co., Tokyo, Japan), with mounted Narishige micromanipulators (Narishige Co. Ltd, Tokyo, Japan).

Before the experiment, 6 drops were prepared under oil in a petri dish cover. A central elongated drop contained the sperm suspension in 20% polyvinylpyrolidone (PVP; 360000 mol. wt, Sigma Chemical Co., St. Louis, MO, USA) in HTF. The second drop contained PVP for pipette washing. Another four drops were HEPES-buffered HTF medium for the oocytes.

The injection pipette was moved to the sperm drop and a single motile spermatozoon was immobilized by pressing its tail with the injection pipette against the surface of the dish and then aspirated tail-first into the tip of the pipette. The oocyte was sucked gently into the holding pipette with

the polar body at 12 or 6 o'clock position (Fig. 2). The injection pipette was quickly inserted through the zona pellucida and the egg membrane until the tip of the pipette was almost to the opposite side of the egg (Fig. 3). A small amount of egg cytoplasm was aspirated into the injection pipette, the cytoplasm and spermatozoon were injected into the egg and the pipette was withdrawn quickly from the egg. The egg was released from the holding pipette and the pipette was withdrawn from the drop.

Experimental protocols

The recovered oocytes were randomized into two groups. In the first group each oocyte was injected by a single tail immobilized spermatozoon. In the second group oocytes were sucked into the holding pipette and released after 1 to 2 minutes and then kept in HEPES-buffered HTF droplets for the time that the other oocytes of the dish were being injected. This control group was chosen to find if the pressure of the holding pipette upon oocytes has a deleterious effect on them.

Assessment of fertilization and embryo cleavage

Following injection, oocytes were incubated in HTF medium at 37° C in an atmosphere of 5% CO_2 in air and examined 6 to 9 hours after microinjection for pronuclear formation. Oocytes with two clear pronuclei and a second polar body were considered to be normally fertilized (Fig. 4). Fertilized oocytes were separated from the rest and examined for cleavage to the 2-cell stage.

Statistical analysis

The results were analyzed by Student's t-test.

RESULTS

The results are summarized in Table I. Of 307 oocytes which were injected with a single spermatozoon successfully, 101 (32.9%) were damaged or degenerated during or shortly after the completion of ICSI; 45.1% of surviving oocytes showed normal fertilization (2 pronuclei and second polar body). The majority of fertilized oocytes cleaved to the 2-cell stage (95.7%).

Of 109 oocytes which were sucked into the holding pipette, 105 (96.3%) survived and only 4 (3.8%) of them became activated parthenogenically. The survival rate in the control group was significantly higher than the study group ($p<0.0001$).

DISCUSSION

The mouse is a good experimental model for ICSI research.[7] However, mouse ICSI is very difficult and gives low survival and fertilization rates due to highly sensitive and small sized oocytes and high elasticity of the previtelline membrane and the long and hook shaped spermatozoon.[8-10] Recently, electrically driven, high speed pipette motion has been used successfully to insert mouse spermatozoa into oocytes and has yielded high survival and fertilization rates.[10] However we think that conventional micromanipulation in mice will be more important if it yields acceptable survival and fertilization rates, because human ICSI has been managed manually in almost all IVF centers. Some investigators succeeded in performing ICSI in mice with conventional methods with reasonably high survival and fertilization rates,[9,11] but unfortunately they did not improve the ICSI method in mice; and it is supposed that their relatively good results reflect mainly their skill in ICSI and some good conditions such as increasing the intracellular concentration of Ca^{++} in oocytes. Ahmadi et al. (1995) got only 8.8% fertilization after intracytoplasmic injection of mouse oocytes by mouse spermatozoa; but they succeeded to increase this rate to 36.5% with injection of Ca^{++} to oocytes before ICSI.[9]

Lacham and Trouson (1995) reported 38% fertilization following mouse ICSI without using Ca^{++}. They talked about a new improved technique for mouse ICSI but unfortunately did not explain the differences between their new technique and the ordinary ICSI method.[11]

In this study, we introduced a new holding pipette which could facilitate mouse ICSI considerably. Using this holding pipette, the oocyte is sucked into a glass tunnel with a diameter less than the mouse oocyte, so the oocyte elongates to a reasonable length; therefore the injection axis will be increased considerably. Due to increase of the injection axis, the operator could pierce the sharp end of the injection needle to a longer distance toward the opposite side of the oolemma and rupture it much easier than the conventional procedure. The fertilization rate was 45.5% using our new holding pipette.

Kimura (1995)
the mouse oocyte membrane did not break even when the pipette tip almost reached the cortex of the opposite side of the oocyte and when a spermatozoon in the pipette was expelled at this position, it seemed as if it had been successfully deposited within the ooplasm, while in reality, it was still outside the ooplasm. We confirm this suggestion and suppose that the approximately low fertilization rates which were reported by some investigators, despite their high survival rates, is reflected to this event partly. This problem could almost be eliminated using our improved method.

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