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# Intracytoplasmic sperm injection in the rat

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## Summary

We applied intracytoplasmic sperm injection (ICSI) to the rat comparing three different sperm injection techniques: conventional setup with a sharp needle bearing a spike (method 1), combination of partial zona dissection (PZD) needle and blunt pipette (method 2) and piezo-injection using a blunt pipette (method 3). We also investigated the timing of sperm pronuclear formation after injection. Survival rates after injection were 8%, 24% and 71% for the methods 1, 2 and 3, respectively. All surviving oocytes formed pronuclei by about 6 h after injection. Although the survival and activation rates following sperm injection using piezo-injection were high, the incidence of normal fertilisation, as evidenced by second polar body extrusion and formation of two pronuclei, was only 10%. The vast majority of the zygotes were multinucleated, although most of them subsequently underwent cleavage. Fixation and staining of injected oocytes at different times after injection revealed that replacement of sperm nuclear protamines by histones takes place by 15 min after injection, sperm head swelling occurs within 0.5–1 h after injection and pronuclei become fully developed by 7 h after injection. Although the rate of normal fertilisation in the rat following ICSI was low under the present experimental conditions, the results indicated that direct ICSI using a piezo-driven pipette would be a potentially valuable method of producing rat offspring.

Keywords: Fertilisation, Intracytoplasmic sperm injection, Oocyte, Parthenogenetic activation, Rat

## Introduction

Most infertile men have spermatozoa in their testes and / or ejaculate. However, in many cases those spermatozoa are incompetent to fertilise an egg, perhaps due to their morphological or biochemical abnormalities. This may prevent transmission of chromosomal aberrations or / and mutations or / and epigenetic deviations to offspring. During intracytoplasmic sperm injection (ICSI) a spermatozoon is injected directly into the ooplasm, rendering any live spermatozoa in the testicle or ejaculate of infertile men capable of participating in fertilisation and thus creating the theoretical possibility of passing adverse factors to offspring. Although this possibility is recognised (Merschede *et al.*, 1995), only a single animal study has been performed to address this issue (Kuretake *et al.*, 1996*a*) because of the technical difficulties associated with injection of spermatozoa in mouse or rat oocytes (Thadani, 1979; Markert, 1983; Barg *et al.*, 1996; Ron-El *et al.*, 1995). While mice cost less than rats, rats have been used more extensively than mice in behavioural tests and reproductive toxicology. In contrast to the mouse, almost no information is available on ICSI in the rat. In a single study dealing with rat ICSI (Thadani, 1979) immature oocytes were used and the oocyte survival rate was only about 20%. Although a relatively high survival rate of rat oocytes following injection of mouse spermatozoa was later achieved, injection of large rat spermatozoa into rat oocytes was extremely difficult (Thadani, 1980). In the present work we report for the first time successful injection of rat sperm into matured rat oocytes.

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## Material and methods

### Chemicals

All chemicals, unless specified otherwise, were purchased from Sigma (St Louis, MO).

### Oocytes

Female Sprague–Dawley rats 23–27 days old were injected intraperitoneally with 20 IU PMSG and 20 IU hCG 48 h apart. They were killed 12–14 h after hCG injection and oocyte–cumulus complexes were recovered from the oviducts into CZB-Hepes medium (Kimura & Yanagimachi, 1995). Cumulus was removed by 10 min treatment with hyaluronidase (ICN Pharmaceutical, St Louis, MO: 300 units/mg). Prior to and after sperm injection, oocytes, in CZB medium, were kept in the incubator at 37 °C in an atmosphere of 5% $CO_2$ in air.

### Spermatozoa

Spermatozoa were collected from cauda epididymides of retired male breeder (Sprague–Dawley) rats. They were allowed to disperse in the CZB for about 5 min and then transferred to the drop of CZB-Hepes containing 12% (w/v) polyvinylpyrrolidone (360 kDa). In order to separate the sperm head from the tail we used one of two techniques. In the case of a conventional setup with one or two pipettes, a pipette was positioned just behind the sperm head and the head separated by clicking down on the microscope stage. In the case of piezo-injection (Kimura & Yanagimachi, 1995) the spermatozoon was aspirated tail-first into the injection pipette and positioned so that the tip of the pipette was in contact with mid-piece. The spermatozoon was then immobilised by aplying two or three piezo pulses.

### Preparation of injection pipette

Injection pipettes were prepared from Drummond glass capillary tubes (Drummond, USA) using a Narishige (Japan) puller, microforge and microgrinder. In some cases a Sutter puller (Sutter, USA) was used. Details of pipette preparation have been described elsewhere (Dozortsev *et al.*, 1996). Briefly, the pipette was broken using a microforge so that its tip had an external diameter of 7–11 µm. Some of the pipettes were bent and used for sperm injection (referred to hereafter as blunt injection pipettes). Other injection pipettes were bevelled at an angle of about 45° and a small- or medium-sized spike made on this needle before it was bent. Needles used for partial zona pellucida dissection (referred to hereafter as PZD needles) were bent at the same angle as a blunt pipette. Holding pipettes had an external diameter of about 100 µm and opening of about 5 µm. The pipettes used for piezo-injection were prepared as above, but without bevelling.

### Micromanipulation

Three different injection methods were used.

*Method 1*
A sharp pipette with a spike was used. The pipette with the aspirated spermatozoon was positioned at 3 o'clock, and inserted deep into the ooplasm, creating extensive invagination of the oolemma. Then the oolemma was aspirated into the pipette until it was broken. A spermatozoon was injected into the ooplasm together with the aspirated ooplasm before the pipette

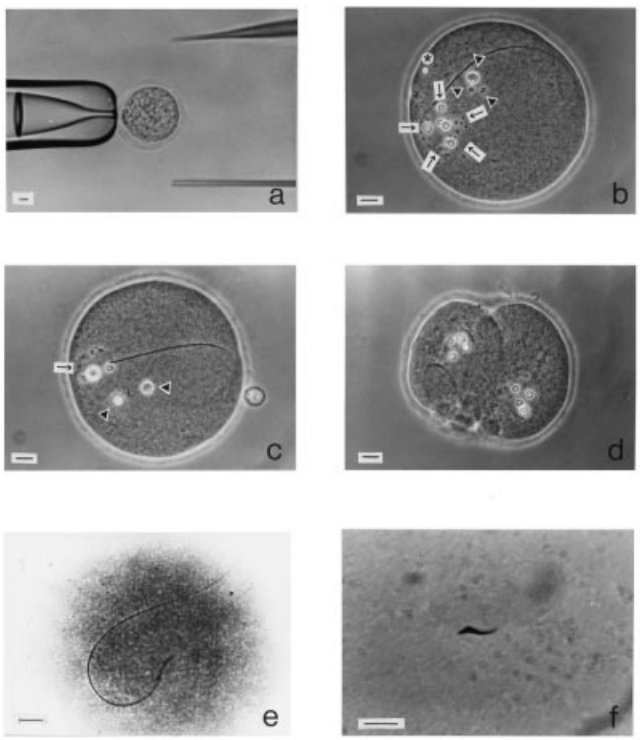



**Figure 1** (*a*) General view of the setup for intracytoplasmic sperm injection using two microtools. Scale bar represents 10 µm. (*b*) Decondensing sperm head with several nucleoli (arrows), female pronucleus (arrowheads) and polar body nucleus (*) 6 h after injection. Aceto-orcein staining. Scale bar represents 10 µm. (*c*) Decondensing sperm head (arrow) and two female pronuclei (arrowheads) 6 h after injection. Aceto-orcein staining. Scale bar represents 10 µm. (*d*) Embryo at the 2-cell stage 26 h after injection. Aceto-orcein staining. Scale bar represents 10 mm. (*e*) Sperm head 5 m in after ICSI has its original size and shape and is not stained by Giemsa. Phase contrast. Scale bar represents 10 µm. (*f*) Sperm head 15 min after injection has its original size and shape but is deeply stained by Giemsa. Scale bar represents 10 µm.

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**Table 1** Oocyte survival and fertilisation following ICSI using different injection methods

| Setup | Type of pipette | Oocytes injected | | | Activated eggs with | | |
|---|---|---|---|---|---|---|---|
| | | Total | Survived | Activated | 2PN + 2PB | 3PN | > 3PN |
| Conventional | Sharp | 35 | 3 (9%) | 3 (9%) | 0 | 1 (33%) | 2 (66%) |
| | Blunt | 38 | 9 (24%) | 9 (24%) | 2 (22%) | 2 (22%) | 5 (56%) |
| Piezo | Blunt | 98 | 70 (71%) | 70 (71%) | 7 (10%) | 19 (27%) | 44 (63%) |

PN, pronuclei; PB, polar bodies.

was slowly withdrawn. After injection the sperm head was clearly visible within the cytoplasm; the tip of the sperm tail protruded outside the zona pellucida.

*Method 2*

A blunt injection pipette was used in combination with a PZD needle (Fig. 1*a*). First, a slit was made in the zona pellucida using a PZD needle. The egg was then positioned with the slit at 3 o'clock. A blunt pipette with a spermatozoon was inserted deep into the oocyte creating an extensive invagination of the ooplasm. From this point on the injection procedure was identical to that described above for the pipette with a spike.

*Method 3*

In this method the piezo-injection approach was used. After the whole body of the spermatozoon had been aspirated into the injection pipette and the sperm head was positioned away from the tip of the opening of the pipette, the pipette tip was brought in contact with the zona pellucida. By advancing the pipette slowly while applying a few piezo pulses the zona was drilled without deformation. The sperm head was then moved to the tip of the pipette and the tip advanced mechanically deep into the oocyte, invaginating the oolemma extensively. The oolemma was punctured by applying one or two pulses. The spermatozoon was then expelled into the ooplasm with a minimum amount (about 6 pl) of accompanying sperm-suspending medium. After as much as possible of the medium had been retrieved the pipette was withdrawn gently, leaving the spermatozoon within the ooplasm (Kimura & Yanagimachi, 1995). The entire operation was performed in CZB-Hepes droplets under mineral oil (Squibb, Princeton, NJ) at room temperature. The gas phase was room air.

### Fixation and staining

To determine how quickly sperm nuclear protamines are replaced by histones, sperm-injected oocytes were fixed at 5–15 min intervals followed by staining with Giemsa (Malaszevski, 1990). By this method, sperm nuclei with S–S bonds between protamines remain unstained, whereas following disintegration of those bonds they become stained dark blue. Cytological details of sperm-injected oocytes were examined by compressing them between a slide and coverslip followed by fixation with 2% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) and staining with aceto-orcein (Yanagida *et al.*, 1991).

## Results

### Oocyte survival and fertilisation following ICSI

Neither blunt nor sharply pointed pipettes could break the oolemma. It was broken by ooplasm aspiration as previously reported by Dozortsev *et al.* 1994). During this aspiration the oolemma was stretched up to 300 µm within the pipette before it was broken. With some rare exceptions, most injected oocytes appeared alive during the 20–30 min after sperm injection. Then, some oocytes swelled as evidenced by a decrease in size of the perivitelline space. They invariably degenerated within next 30 min to 2 h. While only some of the eggs contained two pronuclei (Fig. 1*b*), the majority of activated oocytes contained one large pronucleus connected to the sperm tail and multiple small pronuclei (Table 1). In many cases the extrusion of the second polar body was suppressed (Fig. 1*c*). When the eggs with normal-sized pronuclei reached the 2-cell stage (Fig. 1*d*), the majority of the eggs with multiple small pronuclei were still at the pronuclear stage.

### Transformation of the sperm head

In oocytes fixed 5 min after injection, sperm heads retained their original size and shape. They were not stained with Giemsa (Fig. 1*e*). In oocytes fixed 15 min after injection, sperm heads were darkly stained with Giemsa while they were still their original size and shape (Fig. 1*f*). Between 0.5 and 2 h after injection sperm heads were swollen progressively and detached from tails. Pronuclei were clearly visible under the inverted microscope about 6 h after injection.

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## Discussion

The present study demonstrated that neither a sharp pipette with a spike nor a blunt pipette yielded high survival rates following ICSI in the rat. This is most likely due to the large size of the injection pipette that is required to accommodate the large rat sperm head. However, the large size of the pipette alone cannot be the major cause of high oocyte death rates. We obtained high oocyte survival rates following ICSI using a large blunt pipette driven by piezo-electric force (Table 1). Thus, the method of oolemma breakage appeared to influence the oocyte survival rate. Some additional, not very obvious factors may also contribute to the oocyte survival rate.

Rat oocytes have a high rate of spontaneous activation (Keefer & Schuetz, 1982). Although we did not determine the rate of spontaneous activation in the present study, it is possible that some of the eggs we examined after ICSI were in fact activated parthenogenetically. The causes of spontaneous activation of rat eggs are complicated. However, it is known that the removal of the oocytes from rats immediately before use, as well as maintaining the oocytes *in vitro* at 37 °C, reduces the incidence of spontaneous activation (Gaddum-Rosse *et al.*, 1984; Zernicka-Goetz, 1991).

In the present study only a small proportion of eggs were fertilised normally following ICSI. In many instances the extrusion of the second polar body was suppressed and multiple small pronuclei were formed. This is in agreement with the findings of Thadani (1980), who reported that only about 30% of rat oocytes fertilised by mouse sperm using ICSI had two pronuclei and a second polar body. It has been demonstrated in the rat that following spontaneous activation the oocytes become arrested again (Zernicka-Goetz, 1991) at the so-called MIII stage (Kubiak, 1989), sometimes with chromatids scattered in the ooplasm (Zernicka-Goetz, 1991). We assume that formation of multiple pronuclei is the result of fertilisation after spontaneous activation. The failure of second polar body extrusion after ICSI also takes place in the human when a relatively large injection pipette is used (Dozortsev, unpublished). We believe that in the future we can improve the incidence of successful rat ICSI by preventing spontaneous activation of oocytes in better rat oocyte-handling media.

The present study revealed that reduction of disulphide bonds in protamines begins approximately 15 min after sperm injection. In normally fertilised oocytes reduction of disulphide bonds takes place immediately after sperm entry (Chen & Longo, 1983). The difference is probably due to the fact that during ICSI the whole spermatozoon including the plasma membrane is injected into the ooplasm, and therefore it will take some extra time before the sperm nucleus is exposed to the ooplasm (Dozortsev *et al.*, 1997). The manner of sperm nucleus transformation into pronucleus was similar between *in vitro* fertilised eggs (Gaddum-Rosse *et al.*, 1984) and ICSI fertilised eggs (present study). Rat sperm tail is relatively large and thick. Tails can be detected in rat eggs fertilised *in vitro* at least until after the first mitotic division (Guant, 1983). We could clearly observe sperm tails at the 2-cell stage after ICSI. Injection of the whole spermatozoon into an egg often results in the injection of excessive amounts of micromanipulation medium that may have an adverse effect on subsequent embryonic development (Thadani, 1980). It will be necessary to determine in future whether injection of an isolated sperm head is sufficient for normal development, as in the mouse (Kuretake *et al.*, 1996*b*).

Our preliminary experiments also indicate that rat sperm nuclei injected into oocytes by ICSI about 1.5 h after spontaneous activation remained condensed for many hours. This is in agreement with the findings of Komar (1982), who maintained that ooplasmic factors responsible for sperm nucleus decondensation disappear by 1.5 h after activation.

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