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Fertilization of Eggs of Zebrafish, *Danio rerio*, by Intracytoplasmic Sperm Injection[1]

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ABSTRACT

To evaluate the potential for fertilization by sperm injection into fish eggs, sperm from zebrafish, *Danio rerio*, were microinjected directly into egg cytoplasm of two different zebrafish lines. To evaluate physiological changes of gametes on the possible performance of intracytoplasmic sperm injection (ICSI), four different combinations of injection conditions were conducted using activated or nonactivated gametes. From a total of 188 zebrafish eggs injected with sperm in all treatments, 31 (16%) developed to blastula, 28 (15%) developed to gastrula, 10 (5%) developed abnormally to larval stages, and another 3 (2%) developed normally and hatched. The highest fertilization rate (blastodisc formation) was achieved by injection of activated spermatozoa into nonactivated eggs (35%). Injections were most effective when performed within the first hour after egg collection. Flow cytometric analysis of the DNA content of the developing ICSI embryos revealed diploidy, and the use of a dominant pigment marker confirmed paternal inheritance. Our study indicates that injection of a single sperm cell into the cytoplasm of zebrafish eggs allows fertilization and subsequent development of normal larvae to hatching and beyond.

assisted reproductive technology, embryo, fertilization, gamete biology

INTRODUCTION

Research in intracytoplasmic sperm injection (ICSI) in higher vertebrates began during the late 1970s when hamster ova injected with sperm developed to pronuclei and early cleavage stages [1]. In the past decade this technique has gained interest due to its diverse applications, and it has been evaluated with varying levels of success in different mammalian species [2–4], including humans [5]. In aquatic organisms, only work in echinoderms [6, 7] and amphibians has been reported [8–10]. In the first microinjection experiments in aquatic species, injection of spermatozoa into sea urchin eggs did not trigger activation, cause structural changes in the cortex, nor swelling of the egg nucleus [6]. However, injection of single sperm cells into eggs of the African clawed frog, *Xenopus laevis*, produced normal metamorphosis and development of frogs [8].

It is probable that the variable success found for ICSI depends on intrinsic characteristics of the organism studied. In frogs, fertilization occurs externally and activation of the egg occurs after the sperm has penetrated the jelly coat that surrounds the eggs [8, 11, 12]. Fish spermatozoa, in contrast to mammalian spermatozoa, are immotile when in seminal plasma or in an isotonic or isoionic solution [13]. In nature, the sperm become motile upon entering the external environment (fresh water or salt water), and depending on the species, sperm activation is triggered by changes in osmotic pressure or ion concentration [14]. Sperm capacitation in fish has not been identified, but if it does occur, it may coincide with contact with the external environment. Regardless of the activation mechanism of teleost sperm, they typically have access to the membrane of the egg only through a specific location or sperm entry site (microvillar cluster), beneath an inner aperture called the micropyle [15].

Egg activation begins with a rapid rise in intracellular calcium levels that propagates throughout the egg [16]. In mammals and amphibians, contact with sperm triggers the activation of the egg. In fish, egg activation seems to be species specific. In zebrafish, *Danio rerio*, activation does not occur when sperm react with the egg surface but rather when the eggs come in contact with the spawning medium [17–19]. Thus, activation of eggs could be important when ICSI is performed.

The present study investigated the potential of performing ICSI in teleost fishes using the zebrafish as a model. Our objectives were to 1) evaluate the micropyle as a sperm injection site, 2) evaluate standard ICSI techniques for use in zebrafish, 3) evaluate the effect of gamete conditions (activated or nonactivated) on fertilization and development, 4) evaluate the effect on fertilization of the timing of ICSI after egg collection, and 5) identify ploidy and paternal contribution in ICSI-fertilized embryos.

MATERIALS AND METHODS

Animals

Two lines of zebrafish (pigmented, wild-type, and gold, long-fin) were obtained from a commercial supplier (Scientific Hatcheries, Huntington Beach, CA). The gold, long-fin line is recessive for pigmentation (Dallas Weaver, Scientific Hatcheries, personal communication) that results in a visible lack of pigmentation compared with the wild-type (Fig. 1C). Fish were maintained in 10 separate 80-L aquaria linked in a recirculating system. Filtration was accomplished using an upwelling bead filter. Temperature was maintained at 26°C and a photoperiod of 10D:14L was established. Salinity of the water was maintained at 1.5 ppt by the addition of NaCl (Mix-N-Fine; Cargill, Minneapolis, MN). Hardness (measured as $CaCl_2$) and alkalinity (measured as $NaHCO_3$) were monitored weekly using a standard water quality test kit (model FF-1A; Hach Company, Loveland, CO) and were each maintained at 100 ppm.

Gamete Collection and Preparation

The night before ICSI experiments, adult fish were placed in 6-L breeding tanks in a ratio of three females to two males [20]. Collection of gametes was performed on the day of the ICSI trials. The zebrafish were anesthetized by immersion in water containing 0.17 mg/ml of tricaine methanesulfonate (methyl-*m*-aminobenzoate, MS 222; Argent Laboratories, Inc., Redmond, WA). Sperm were obtained by gently squeezing the

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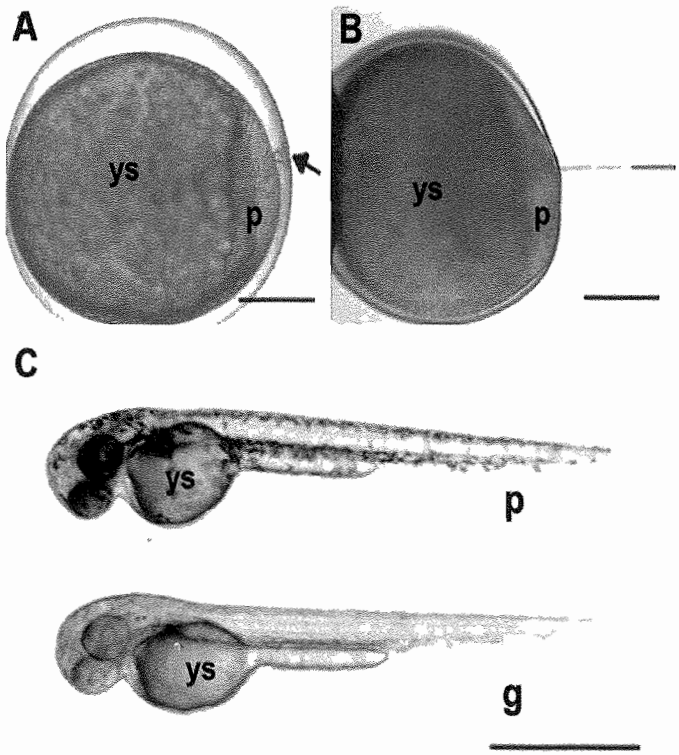

FIG. 1. **A**) Activated zebrafish egg. The micropyle is indicated by the arrow. **B**) Nonactivated zebrafish egg after microinjection with sperm: p, animal pole; ys, yolk sac. **C**) Zebrafish embryos at 48 h after fertilization: wild-type, pigmented (p), and gold, long-fin zebrafish (g). Scale bar in **A** and **B**, 200 μm; **C**, 1 mm.

sides of the males followed by dilution in Hanks balanced salt solution (HBSS) [20] and storage on ice until use. Eggs were obtained by gently pressing on the sides of females, starting behind the pectoral fins, and moving toward the tail. Nonactivated eggs were kept in HBSS containing 0.5% BSA [21] until use. The first 35 injection trials in this study were used to establish basic techniques. These trials were not included in the analysis of injection time after egg collection (n = 153); however, they were included in the overall results (n = 188).

Activation of gametes was induced by transferring them to embryo medium buffer (EMB) (13.7 mM NaCl, 5.40 mM KCl, 0.25 mM Na_2HPO_4, 0.44 mM KH_2PO_4, 1.30 mM $CaCl_2$, 1.00 mM $MgSO_4$, 4.20 mM $NaHCO_3$) [20] at 52 mOsmol/kg. Gamete activation was analyzed in a 2 × 2 factorial design comparing the use of activated and nonactivated sperm and eggs. Eggs from individual females were assigned across treatments and sperm from pigmented males only were used in all experiments.

Sperm Injection

Injections were performed following accepted methods for ICSI in other vertebrates [22] with some modifications described below. Eggs were placed in 100 μl of EMB or HBSS in the lid of a 100-mm plastic cell culture dish (Corning Glass Works, Corning, NY) and covered with mineral oil. Microinjections were performed using an inverted microscope (Diaphot Nikon Inc., Tokyo, Japan) equipped with two mechanical micromanipulator units (Leitz, Rockliegh, IL). Injecting pipettes (~15 μm internal diameter) and holding pipettes (~300 μm internal diameter) were made from borosilicate glass capillary tubes. A sharp spike was created at the tip of the injecting pipette.

Nonactivated sperm were diluted in HBSS containing 5% polyvinylpyrrolidone (PVP) (360 000 M_r) (Sigma Chemical, St. Louis, MO) that was used to prevent the sperm from sticking to the inner wall of the injection pipette. Activated sperm were produced by dilution in EMB containing 5% PVP. Zebrafish sperm became motile after contact with the EMB and lost motility within 60 sec of activation. The activated sperm used for injection were injected in a nonmotile condition. A single sperm was injected with ~7 pl of injection buffer (HBSS plus 5% PVP or EMB plus 5% PVP depending of the treatment). For injections, the egg was held with the animal pole facing outward as the injection pipette was pushed through the micropyle into the cytoplasm (Fig. 1B).

Zebrafish eggs undergo many physical changes when activated. One is the expansion of the chorion that causes detachment of the micropyle from the egg cytoplasm. Injections in activated eggs were performed only when the micropyle was connected to the egg plasma membrane. After injection, eggs were transferred immediately to a 35-mm tissue culture dish (Corning Glass Works) containing EMB and were incubated at 28°C. After 3 h, the eggs were inspected under a dissecting microscope for development. Embryos that presented a well-developed blastodisc were counted as fertilized and were left for further development at 28°C.

Two different sets of control treatments were performed. To evaluate the possibility of parthenogenetic activation by piercing (e.g., gynogenesis), eggs were injected only with injection buffer. The second control was done to observe possible damage to embryonic development caused by injection and toxicity of PVP. Because the incorporation of sperm into the zebrafish egg cytoplasm occurs within 2–3 min of insemination [15], eggs were injected with ~7 pl of EMB containing 5% PVP at 5 min after artificial insemination using fresh sperm (no ICSI).

Egg quality was evaluated for each experiment (for each female) by observation of the percentage of fertilization by artificial insemination. A known number of stripped eggs were incubated with fresh sperm in 500 μl of EMB. After 10 min, 4 ml of EMB was added, and the eggs were incubated a 28°C. Percentage fertilization and development were verified at 3 h and at 24 h after insemination. Embryos that presented a well-developed blastodisc at 3 h after artificial insemination were counted as being fertilized.

DNA Content Analysis

Analysis of DNA content was performed using a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA) equipped with an air-cooled 480-nm argon laser. The FACSComp software (Becton Dickinson) was used to calibrate the instrument. Embryos were disrupted by passage through a 3-ml syringe fitted with a 25-gauge needle while suspended in 0.5 ml of lysis buffer containing 25 μg buffered RNase, 0.1% sodium citrate, and 0.1% Triton X-100 [23]. Samples were mixed and filtered through 20-μm nylon mesh. For analysis, an aliquot of 250 μl was diluted in lysis-staining buffer that included 25 μg of propidium iodide. A sample of frozen blood from rooster, *Gallus gallus*, was used as an internal reference (2.5 pg of DNA/cell) [24]. Measurements of the DNA content of normal diploid zebrafish (3.15 ± 0.06 pg of DNA/cell) and haploid zebrafish (1.59 pg) were compared with those of larvae produced by ICSI.

Statistical Analysis

Differences in the incidence of fertilization among treatments were evaluated using a logistic regression [25]. The statistical model used was:

$$\log P/(1 - p) = \alpha + \beta_1 S + \beta_2 E$$
$$= -1.439 + 0.8224\ (S) + (-1.0821)\ (E) = a$$

where α is the intercept; β_1 and β_2 are the logistic regression coefficients; S is sperm (designated as 1 for activated sperm or 0 for nonactivated); E is eggs (designated as 1 for activated or 0 for nonactivated). Probabilities of fertilization were calculated by:

$$p = e^a/(1 + e^a)$$

where p is the probability of fertilization and is the value predicted by the model. Differences were considered to be significant at $P < 0.05$.

RESULTS

Sperm Injection Site

Localization of the micropyle, before activation or when the eggs were suspended in extender solution, was difficult because there was no clear demarcation between the animal and vegetal poles. This difficulty in distinguishing the position of the animal pole while in extender solution increased the difficulty of localization of the micropyle in nonactivated eggs. Also due to the size of the eggs (~800 μm) and dense cytoplasmic content, the tip of the injection

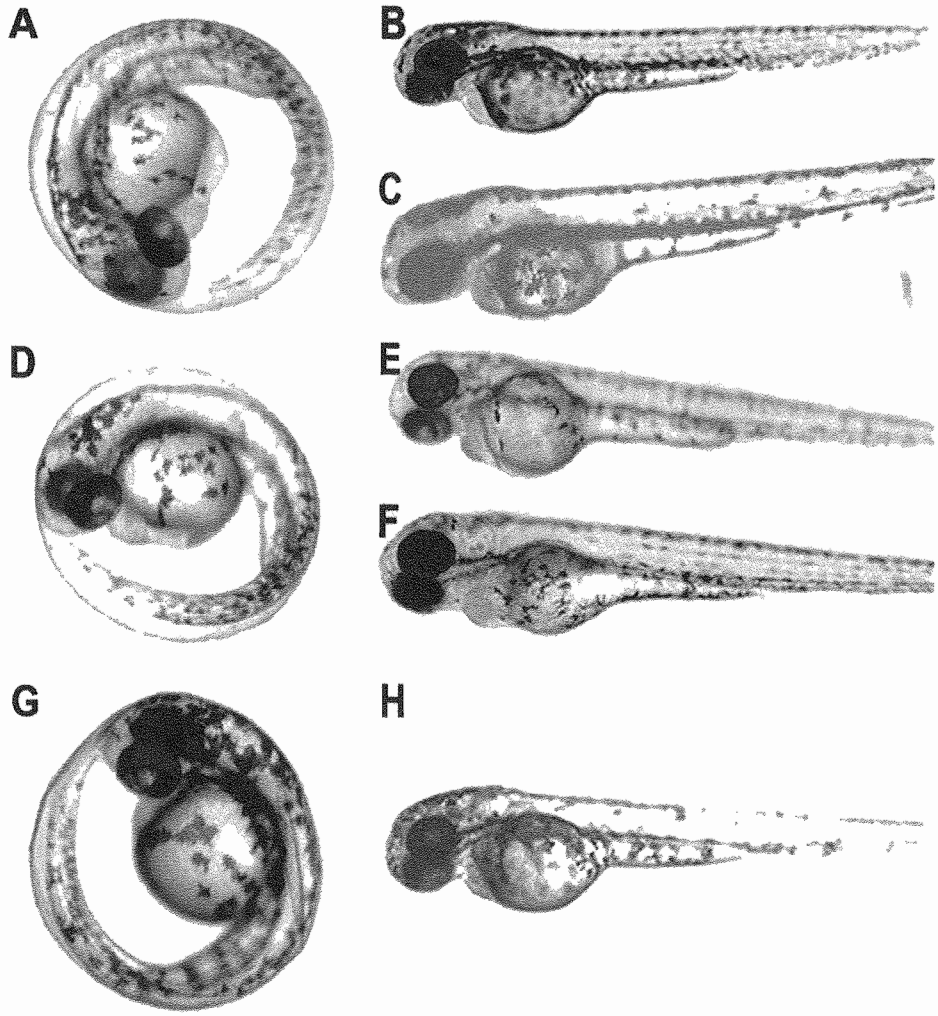

FIG. 2. Comparison of embryonic stages between normal zebrafish embryos obtained by artificial insemination (**A–C**) and embryos produced by ICSI (**D–H**). ICSI embryos obtained by injecting sperm from wild-type, pigmented males into eggs from wild-type, pigmented females (**D**). Fully developed embryos inside the chorion (48 h after fertilization) (**A**, **D**, **G**). Hatched embryo (56 h) (**B**, **E**, **H**) and 5-day-old larvae (**C**, **F**). Zebrafish embryos produced by injecting sperm from wild-type, pigmented males into eggs from gold, long-fin females (**G–H**). The resulting fish show the dominant pigmented phenotype.

pipette was sometimes not visible inside the eggs. This created difficulty in monitoring of the injection of the sperm into the egg.

Gamete Conditions

The different gamete treatments were evaluated using eggs from two different zebrafish lines (gold, long-fin and wild-type, pigmented) and sperm from wild-type pigmented males. There was no significant difference in fertilization by ICSI between the two zebrafish lines ($P = 0.997$; logistic regression), and therefore, these results were combined for further analysis (Table 1). Overall, there was no difference in fertilization ability of activated and nonactivated spermatozoa by use of the ICSI procedure ($P = 0.057$). However, there was a significant difference in fertilization between activated and nonactivated eggs ($P = 0.010$). From a total of 188 eggs injected with sperm, 31 (16.5%) developed into blastulae, 28 (14.9%) developed into gastrulae, 10 (5.3%) developed abnormally as larvae, and 3 (1.7%) developed normally and hatched (Fig. 2). Two of the normal fish were killed for analysis of DNA content at 7 days after hatching. The third fish grew to begin feeding (7 days after hatching) and died when the holding tank was accidentally drained. The probabilities of fertilization calculated for the different treatments were 35% when activated sperm were injected into nonactivated eggs, 19.2%

TABLE 1. Effect of sperm injection using activated and nonactivated gametes.[a]

	Sperm			
	Activated		Nonactivated	
Eggs	Number	%	Number	%
Activated				
Total injections	83	100.0	56	100.0
Fertilized (blastodisc)	13	15.7	4	7.1
Blastula	13	15.7	4	7.1
Gastrula	12	14.5	4	7.1
Abnormal embryos	5	6.0	1	1.8
Normal embryos	1	1.2	1	1.8
Nonactivated				
Total injections	29	100.0	20	100.0
Fertilized (blastodisc)	10	34.5	4	20.0
Blastula	10	34.5	4	20.0
Gastrula	9	24.1	3	15.0
Abnormal embryos	4	13.8	—	—
Normal embryos	—	—	1	5.0

[a] Only sperm from wild-type pigmented males were injected into eggs of two zebrafish lines (wild-type, pigmented and gold, long-fin). No significant difference was observed in fertilization rate between the two different lines ($P = 0.997$, logistic regression) and the data were combined. All embryos that developed after 48 h were pigmented.

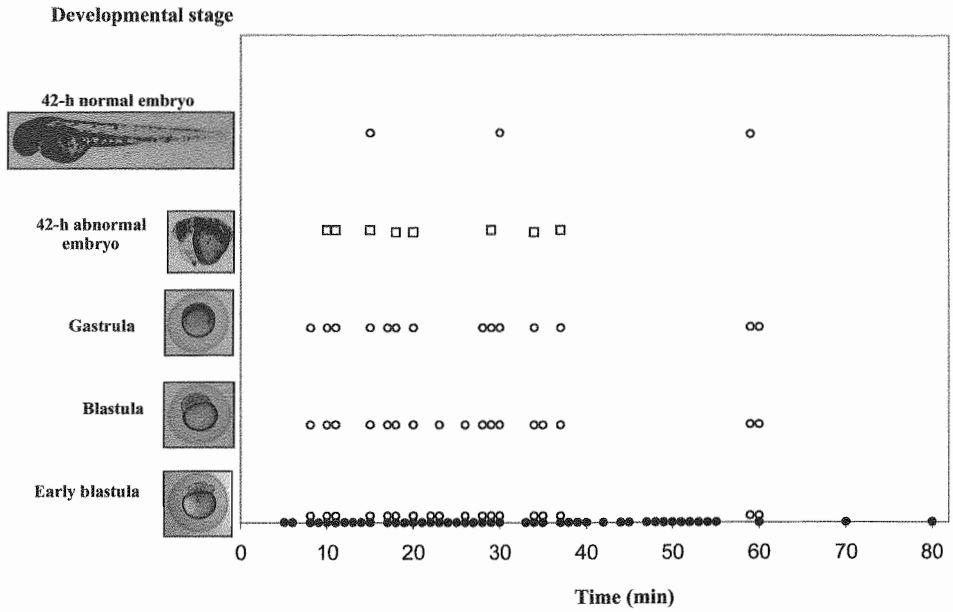

FIG. 3. Effect of elapsed time after egg collection on fertilization rate of ICSI (n = 153). The x-axis represents the time elapsed between egg collection and sperm injection. The developmental stages are depicted on the y-axis. Filled circles indicate unfertilized eggs after ICSI. Open circles indicate fertilized eggs. Squares indicate embryos that showed developmental abnormalities.

when nonactivated sperm were injected into nonactivated eggs, 15.5% when activated sperm were injected into activated eggs, and 7.4% when nonactivated sperm were injected into activated eggs.

Of the 21 eggs injected only with injection buffer (EMB plus 5% PVP or HBSS plus 5% PVP), none developed beyond the one-cell stage, and thus none met the criteria for fertilization. Of the eight eggs injected with injecting medium after artificial insemination, seven (89.5%) developed normally to hatching, and one did not develop beyond the one-cell stage. This was not different from the overall fertilization percentage (62.0% ± 31.0%) that we observed for the 4200 control eggs that were artificially fertilized (eggs of ~42 females).

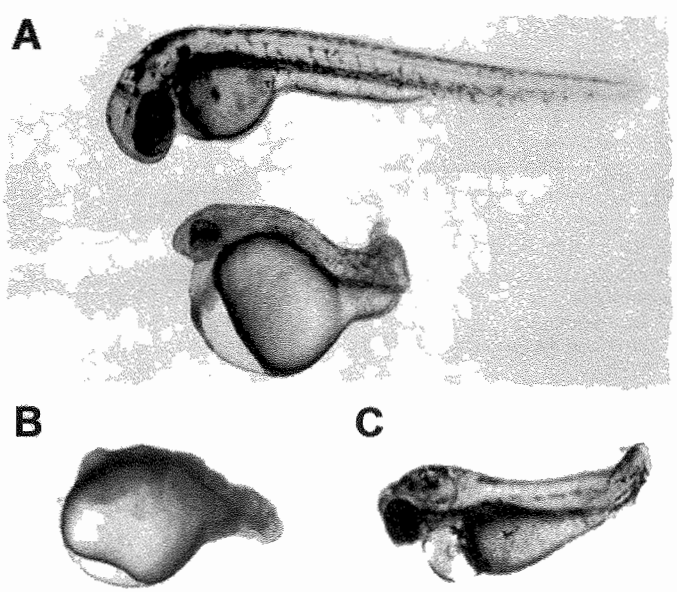

FIG. 4. Abnormal development of zebrafish embryos after ICSI. **A**) Normal embryo (top) at 48 h after artificial insemination and abnormal embryos (bottom) at the same time after ICSI. **B**) Headless 24 h after ICSI. **C**) Abnormal larvae (72 h after ICSI) showing pigmentation produced by injection of sperm from pigmented males into eggs from gold females.

Effect of Time after Egg Collection

The time after egg collection was recorded for the injection of 153 eggs. Injections resulted in 27.7% fertilization in the first 20 min after egg collection, 18.0% fertilization from 21 min to 40 min after egg collection, and 9.1% fertilization from 41 to 60 min after egg collection (Fig. 3). With respect to ICSI, normally developed, hatching larvae were produced from eggs injected at 15, 21, and 60 min after egg collection.

Development

Ten of the 13 ICSI embryos that developed beyond the gastrula stage were abnormal. All 11 embryos that survived beyond 48 h showed wild-type pigmentation, and all of the abnormal embryos showed pigmentation as well, indicating successful incorporation of the male genome. The abnormal zebrafish showed signs of developmental retardation (Fig. 4). The anteroposterior axis was compromised as indicated by a curvature of the caudal fin (Fig. 4, A and C). The eyes were poorly developed and were smaller than normal. The yolk sac in abnormal embryos persisted longer and thus appeared to be larger than that of normal fish (Fig. 4A). DNA content of the abnormal larvae was 3.1 pg of DNA per cell indicating that they were diploid.

DISCUSSION

The present work evaluated the possibility of applying standard ICSI technology to fishes. Basically the same equipment used for ICSI in mammals was used in zebrafish. However, the size of the zebrafish eggs dictated the sizing of the injecting and holding pipettes and affected ease of manipulation. The zebrafish egg has a diameter of ~800 μm comprised mostly by a yolk sac in the vegetative pole. This contrasts with the smaller overall size of mammalian ova (e.g., the human ovum is ~100 μm diameter), although it is important to note that the animal pole of the zebrafish presents a comparably sized target (~100 μm) for injection (Fig. 1A).

In a condition typical for most fishes, fertilization occurs only at a single location (the microvillar cluster) [15], which is located beneath the micropyle. Because sperm nor-

mally traverse the micropyle to gain entry to the egg, we selected it as the path for the injection pipette. This differs from ICSI in mammals where piercing of the egg is not limited to a specific location of the zona pellucida. Although the micropyle is situated at the animal pole, it was difficult to localize in zebrafish eggs immediately after collection. However, after 10–15 min in extender solution or after egg activation, the border between the poles was better differentiated and localization of the micropyle became easier.

In fishes that spawn by external fertilization, sperm capacitation (if it occurs) would coincide with contact with water and activation of motility by changes in osmotic pressure or ion concentration. This mechanism suggested that fish sperm would need to be activated before injection into the egg. Our results show that activation of sperm was not a prerequisite for fertilization by ICSI. However, we observed a significantly higher probability of fertilization when activated sperm were injected into nonactivated eggs (although, we cannot exclude the possibility that partial activation of the eggs occurred while in the extender). We also observed fertilization when nonactivated sperm were injected into activated eggs. In addition, the eggs had a limited time period (1 h or less) during which fertilization was possible after collection.

Although there are many reports of successful ICSI in humans and domestic animals, the technique is also associated with failures and reduced developmental competency of embryos [26–30]. This was observed in our study as abnormal development and low fertilization rates occurred after ICSI. While it is necessary to note that these were preliminary trials intended only to evaluate the technical potential for ICSI in fishes (improved techniques await development), there are several biological possibilities that must be considered. One is the possibility that abnormal embryos were haploid carrying only a maternal genetic contribution. However, the phenotypes observed did not correspond with those shown by the haploid syndrome in zebrafish [31]. Also, flow cytometric analysis of nuclear DNA content confirmed diploidy. The injection of sperm with a dominant pigmentation marker provided additional proof that the diploid embryos resulted from fertilization by the injected sperm and were not derived through gynogenesis (e.g., parthenogenesis resulting in all-maternal inheritance).

Other explanations of the abnormalities that resulted after ICSI include that the injections may have disrupted a meshwork of actin filaments in the fertilization cone just beneath the plasma membrane of the sperm entry site [32]. It has been suggested that these filaments are responsible in zebrafish eggs for the stabilization of the microvilli cluster of the sperm entry site, for the formation of the fertilization cone, for the binding and fusion of the sperm plasma membrane with the microvilli, and for the movement of the sperm nucleus into the inner cytoplasm [32]. We also cannot rule out the possibility of damage to the egg chromosomes by the injections. It is known that the second polar body is extruded in the vicinity of the micropyle after fertilization [15], indicating the presence of maternal chromosomes in that region. However, eggs that were injected only with injection buffer after artificial insemination did not show abnormal development. Future studies should address the effect of disruption in this area, the development of procedures to minimize injury to the injection site, and evaluate the potential for injection in other sites.

The development of ICSI in fishes could yield valuable applications. The use of cryopreservation of sperm will become a standard practice in fishes in the near future [33]. Although useful, cryopreservation can be costly, and developing countries or small facilities may not be able to afford long-term storage in liquid nitrogen. Alternatives include the use of freeze-dried sperm samples [34] or those preserved in alcohol [35]. These sperm would not need to be motile if ICSI procedures were available to produce fertilization of fish eggs. Given the technical requirements for ICSI, the procedure would (with present technology) be best suited for reconstitution of desired lines and the production of small broodstock populations to be used to produce large numbers of fish. This approach could have application in restoring endangered or extinct stocks by injection of cryopreserved sperm into irradiated eggs from a related stock or species to produce androgenesis (all-paternal inheritance of nuclear DNA) [36, 37].

Other applications could be in the production of transgenic fish. One problem in gene transfer in fishes is the occurrence of mosaicism (incomplete incorporation of the transferred DNA in various cells or tissues). A new approach in mice [38] and the African clawed frog [9, 10] has used ICSI to inject membrane-disrupted sperm heads that are briefly incubated with linearized DNA. This method could yield stable and uniform gene transfer in fishes.

In conclusion, various combinations of conditions of the gametes allowed fertilization by ICSI. However we suggest that sperm injection be pursued using nonactivated eggs and activated sperm. This combination yielded the highest probability of fertilization and the use of nonactivated eggs allowed more time to position the eggs before injection. Our data also suggest that injections will be more likely to fertilize an egg at 5–40 min after collection. Thus, although we were able to induce fertilization and embryonic development by sperm injection in zebrafish, the efficiency was low. Improved methods of injection are necessary and identification of specific constraints will assist understanding of ICSI and the fertilization process in fish eggs.

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