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Original Article

Electrically fused-egg induction and its development in the goldfish, *Carassius auratus*

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ABSTRACT In this study, we report the induction of cell fusion between two fertilized eggs and viable chimeric fish from the fused egg in goldfish. Chemical fusion by polyethylene glycol between denuded fertilized egg induced egg adhesion but rarely egg fusion. This treatment seemed not suitable for egg fusion, because of tight adhesion between vitelline membrane and surrounding matrix. Electrically fertilized egg fusion was successfully induced at the rate of almost 100% under conditions of 400V/cm, 1 MHz dielectrophoresis pulse for 3 seconds and a successive, 750V/cm, 10 μsec fusion pulse. The treatment had to be applied to the egg in the calcium-free low electrolyte medium within 20 min of fertilization at 20°C. Small numbers of fused eggs developed into young chimeric fish, and the different cells originating from two eggs survived together as an individual.

KEY WORDS: *goldfish, electric cell fusion, chimeric fish*

Introduction

Cell fusion has provided many new possibilities in cell manipulation, such as monoclonal antibody production. The application of cell fusion in denuded plant cells (protoplast) has produced new plant breeds. The single plant cell has the potential to develop into an individual under appropriate culture conditions. This ability is called «totipotency». While in the higher animals only female mature germ cells and fertilized eggs have this potential, some kinds of culture cells called «embryonic stem cells» also have it. To date, there have been few experiments applying cell fusion techniques to totipotent fertilized eggs. Successful examples of cell fusion applied to the egg involve nuclear transplantation in mammalian species, (murine, McGrath and Solter, 1983; ovine, Willadsen, 1986; bovine, Prather *et al.*, 1987). These techniques indicate clear totipotency and pluripotency of nucleus, functional difference between female and male pronuclei, and nucleo-cytoplasmic interaction during early development (McGrath and Solter, 1984; Howlett *et al.*, 1987). In fish, as in amphibians, nuclear transplantation has been attempted by microinjection (Tung *et al.*, 1963; Yan, 1989), not by cell fusion.

The authors reported dechorionation in *Carassius auratus* (Yamaha *et al.*, 1986), exotic reagent introduction to denuded eggs by electroporation (Yamaha *et al.*, 1988a), and visualization of the pronucleus and zygote nuclei by Hoechst 33342 vital staining (Yamaha *et al.*, 1988b). Denuded eggs, like protoplast, are useful material for manipulation of fish embryos.

Cell fusion has been induced by biological, chemical, and physical procedures. Biologically, HVJ, named Sendai Virus, induces cell fusion and is used in nuclear transfer in mammals. Chemical fusion by polyethylene glycol or polyarginine does not need any special structure on cell surface but the toxic influences are quite strong (Okada, 1976). Physical cell fusion is induced electrically and is called electric cell fusion. This fusion phase includes two steps: the first step is dielectrophoresis by high frequency pulses, which causes the loss of the membrane charge and the polarization of the dissociated cells, resulting in the attachment of the two cell membranes electrically: the following step is higher pulse treatment, which breaks the attached membrane and induces fusion (Zimmermann and Vienken, 1982).

There are several possibilities, such as nuclear transplantation, for applying the cell fusion technique to the fish egg. In the present study, we report the induction of cell fusion between two fertilized eggs and viable chimeric fish from the fused egg, using chemical and electric cell fusion techniques.

Results

Chemical egg fusion

Several eggs adhered to each other with random directions of blastodisc. The frequency of egg fusion was less than 0.1%. Two eggs adhered to each vitelline membrane, but histological sections revealed that the two eggs did not fuse with their egg cytoplasm. In order to fuse the eggs in the oriented direction, two eggs were set in a 1.6 mm-diameter well made in the plastic dish and 50% PEG

Abbreviations used in this paper: PEG, polyethilene glycol.

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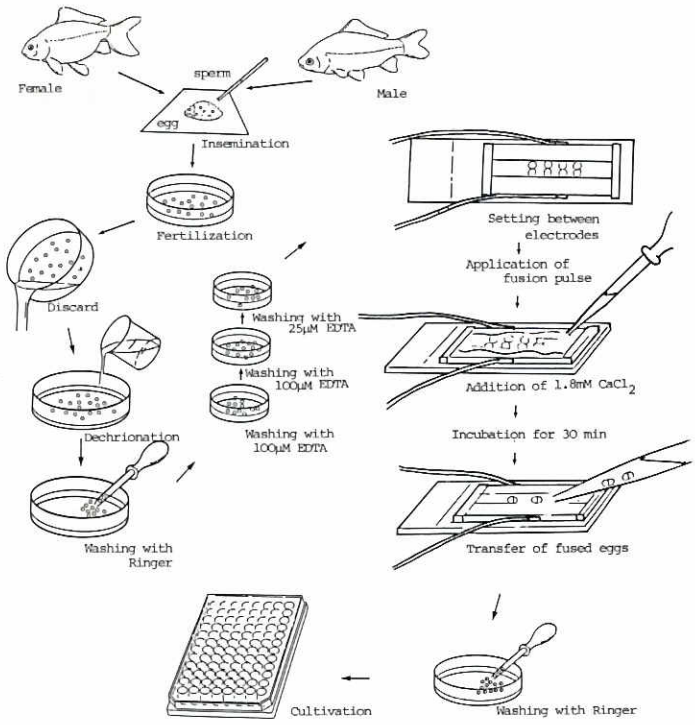

Fig. 1. Schematic illustration of electric cell fusion between two fertilized denuded eggs.

solution was poured over the eggs. The eggs soon adhered to each other but also adhered to the plastic dish matrix. This adhesion was also observed between the egg and the surrounding matrix, including plastic, glass or several kinds of metals. It was difficult to detach the adhered egg from the matrix. This adhesion caused the destruction of paired eggs. The PEG solution treatment had a harmful effect on cleavage.

Electric egg fusion

Egg fusion was induced electrically and the appropriate condition was 400V/cm, 1 MHz dielectrophoresis pulse for 3 seconds and a successive, 750V/cm, 10 μsec fusion pulse.

Four types of fusion instruments were applied to the electric cell fusion (ECF 2001, Riko Kagaku Kenkyusho; ESCF-3001P, Denshi Kagaku Co. Ltd.; SSH 1 and SSH 10, Shimazu Seisakusyo Co. Ltd.). Three of them, however, supplied too weak a dielectrophoresis pulse, even at maximum strength, to cause egg adhesion of satisfactory width. The SSH10 instrument had enough dielectrophoresis pulse power for egg fusion.

The fusion procedure is illustrated in Fig. 1 and the fusion processes are shown in Fig. 2. In the fusion conditions, described above, all egg pairs fused together to become a single egg. The axes of the fused eggs were random, and various types of fused eggs were obtained (Fig. 3b-d). When several eggs were close to each other in the 2 mm space between electrodes, all the eggs fused to become one egg with one vitelline membrane (Fig. 3e).

Although egg fusions were successfully induced under these condition, there were several cases when the egg fusion was not induced or other factors inhibited egg fusion.

The agar-like substance which existed in the perivitelline space interrupted the egg attachment and fusion when the dechorionation treatment was incomplete. As these substances were bound to the several lectins combined to FITC (E.Y. Laboratories), they seemed to contain several sugars, and to be derived from cortical alveoli.

When the fusion pulse was applied to the eggs, egg constriction along with the electric field direction was observed according to their strength. When wider and stronger pulses were applied, the constriction occurred more severely and rapidly, and the eggs were flattened parallel to the electrodes. The strong constriction occurred in the medium containing calcium ion. It was also observed on pulse application during blastodisc formation from 20 min after fertilization (20°C) without calcium ion. The strong constriction destroyed the cytoplasmic bridge between two eggs and inhibited the egg fusion. So it was necessary for the fusion pulse to be applied to the eggs within 20 min of fertilization under calcium-free conditions. In contrast, fusion was easily induced between two blastodiscs, not between the yolk bodies, of denuded unfertilized eggs with slightly deformed blastodiscs, which were left for over 8 h.

It was difficult to suppress this constriction with the treatment of reagents, such as cytochalasin B, nocodasol and colchicine, which inhibit the polymerization of cytoskeletal proteins. Cold treatment at 0°C by ice had no effect on the constriction, but it delayed blastodisc formation and prolonged the applicable time for successful egg fusion.

One fusion pulse was sufficient to fuse eggs. More than one fusion pulse also induced egg constriction and breakdown of the cytoplasmic bridge of the paired eggs by dielectrophoresis pulse. The strength and duration of dielectrophoresis pulses were more important than their frequency. Less than 400 V/cm dielectrophoresis pulse did not make a satisfactory width of contact between eggs. The stronger, longer dielectrophoresis pulses expelled the eggs from between the electrodes and destroyed them.

Development of fused eggs

The fused eggs were classified into the following three types; 1) with two blastodiscs located close together (35%, 77/218, Fig. 3b), 2) with two blastodiscs located independently (19%, 42/218, Fig. 3c), and 3) with one or two blastodiscs placed between two yolk bodies (45%, 99/218, Fig. 3d). The last type of fused eggs developed abnormally and did not survive beyond feeding stage. The development of the first and the second types is shown in Fig. 4. All the second type had two embryonic bodies, and each embryo held one yolk sac, which connected to the other, at the hatching stage (Fig. 5c,d). A small number of these parabiotic fish were able to feed but not to swim. The first type of eggs developed into normal embryos with one embryonic body (16%, 13/77) or parabiotic embryos with two embryonic bodies. All the parabiotic embryos, which were different from the second type, held one yolk sac at the hatching stage but died before reaching feeding larva stage (Fig. 5b). The normal larvae with one embryonic body from fused eggs were about 1.5 times larger in size than control ones. Therefore, they fed easily and grew rapidly after absorption of the yolk. There survived only three normal fish developed from the fused eggs between two strains with pigmented and nonpigmented clear scales (Fig.6). Clear scales were distributed on one side of all these fish. One of them had a black eye without guanine, which originated from the strain with clear scales.

When there were infrequently fused eggs between unfertilized and fertilized eggs, the blastodisc of the unfertilized egg was broken during the cleavage stage within 12 h of fusion.

Discussion

Egg fusion in sea urchin was induced chemically by polyarginine (Bennett and Mazia, 1981) and physically by electric fusion (Richter *et al.*, 1981). Chemical egg fusion, in *Strongylocentrotus purpuratus* and in *Lytechinus pictus*, was induced at the rate of 40% or 5% in unfertilized egg, and 35% or 15% in fertilized egg, respectively (Bennett and Mazia, 1981). Electric unfertilized egg fusion in *Paracentrotus lividus* was induced at the rate of 60% (Richter *et al.*, 1981). Treatment procedures were more complicated in chemical fusion than in electric fusion. Chemical and physical fusions were attempted with the denuded fish egg in this study. Chemical fusion procedures using polyethylene glycol (PEG) induced adhesion of not only egg to egg, but also egg to matrix. As the adhesion between egg and matrix was irreversible, eggs had to be treated by PEG under the floating condition without attachment to the matrix. In order to induce fusion, other successive steps may be needed, such as high concentration of calcium ion or hypertonic shock. On the other hand, electric fusion was successfully induced. In goldfish eggs, electric fusion is easier and more reliable than chemical fusion, although the instrument of electric cell fusion is expensive.

A few problems still remain in electric egg fusion. We used four kinds of instrument for the electric cell fusion in this study. However, three of them did not have enough dielectrophoresis pulse to induce egg attachment. Fusion rates decreased to about 0% under such weak dielectrophoresis pulse conditions or it took longer (2-6 h) to fuse into one egg body. It is said to be difficult to increase the strength of dielectrophoresis pulse at the same conditions as pulse frequency (1 MHz). There was a mechanical limitation of some hardware in fish egg fusion in these instruments. Finally, electrical fertilized egg fusions were successfully induced at the rate of almost 100% at conditions of 400V/cm, 1 MHz dielectrophoresis pulse for 3 seconds and a successive, 750V/cm, 10 μsec fusion pulse.

The fusion pulse has to be applied to the egg within 20 min of fertilization at 20°C, and the egg polarity was not easily detected during this time. The two blastodiscs are located in random positions in the fused eggs. For nuclear transplantation or viable chimeric fish production, it is necessary to develop appropriate methods, such as vital staining, for easily detecting the animal pole.

The egg constriction was observed on application of the fusion pulse and the strong one destroyed the cytoplasmic bridge between eggs, resulting in the separation of the two eggs. This constriction was also observed on electroporation (Yamaha *et al.*, 1988a). Although this reaction was not reported concerning electric cell fusion processes in sea urchin (Richter *et al.*, 1981), the constriction was observed just after fusion pulse in star fish eggs, when electric cell fusion was applied (Yamaha, unpublished data). Markert and Petters (1977) observed that a wave of disintegration sometimes radiates from the wound, when the tip of the micropipette was inserted into the egg cytoplasm in mouse. They thought it was caused by the contraction of microfilaments. From this study in goldfish, it seems that there are at least two kinds of constriction to the fusion pulse. The first pulse constriction within 20 min of fertilization was suppressed by washing with calcium-free or weakly chelated medium, and the second constriction during blastodisc formation could not be suppressed even in the medium containing some depolymerizing reagent for cytoskeleton, such as cytochalasin B or nocodasol, which caused actin filament and microtubule depolymerization, respectively. Elinson (1983) reported the alteration of animal half cytoplasm of the first cell cycle in *Rana pipiens* and *Xenopus laevis* and divided the cell cycle into four cytoplasmic phases. Phase 1 indicates the events of activation and lasts about one-third of the first cycle. In phase 2, the cytoplasm becomes fluid

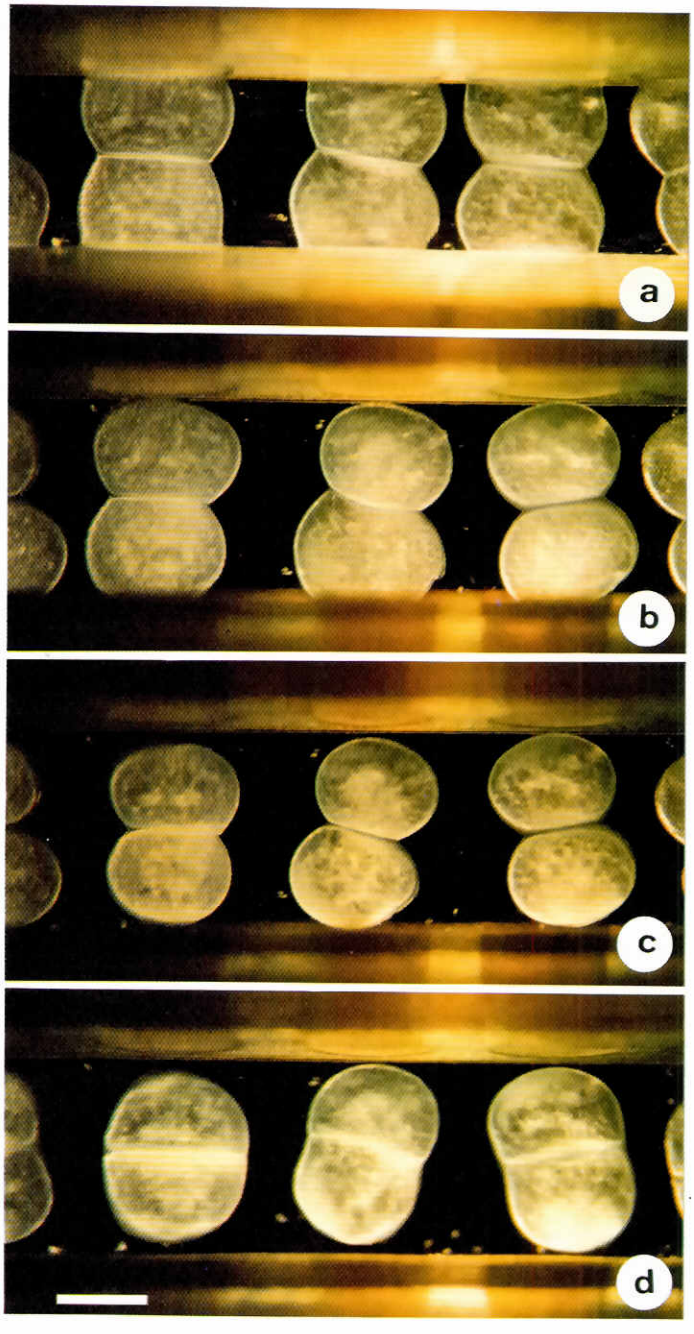

Fig. 2. Actual process of electric cell fusion. (a) *Treatment with dielectrophoresis pulse.* **(b)** *Soon after fusion pulse.* **(c)** *15 minutes after fusion pulse.* **(d)** *30 minutes after fusion pulse. Bar indicates 1 mm.*

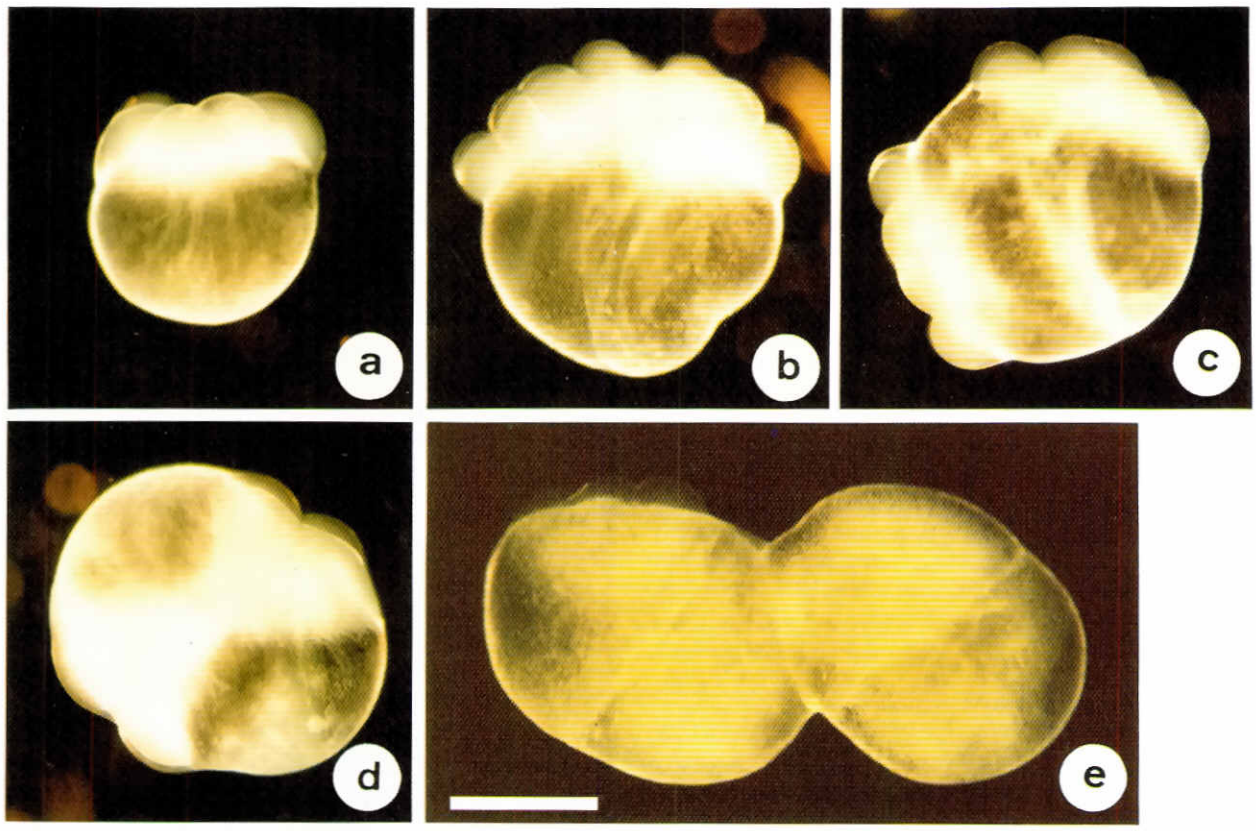

Fig. 3. Fused eggs classified by their blastodisc locations. (a) *Control egg;* **(b-e)** *fused eggs. Bar indicates 0.5 mm.*

except for the rigid growing sperm aster, and most of the migration of pronuclei occurs. In phase 3, the cytoplasm becomes firm and forms the grey crescent. The firmness of the cytoplasm becomes sensitive to colchicine but not to cytochalasin B. In phase 4, the cortex detaches itself from the firm cytoplasm, which is now cytochalasin B sensitive and colchicine insensitive. In goldfish eggs, activation and secretion from the cortical alveoli take place within 10 min of fertilization which corresponds to phase 1 in amphibians. The whole egg becomes firmer from 20 min after fertilization, which corresponds to phase 3 in amphibians. The appropriate time of fish egg fusion may correspond to phase 2, because the whole egg is in the soft and fragile stage in the first cycle. Cytoplasm and yolk separated from each other during blastodisc formation. The firmness of yolk body was not sensitive to cytochalasin B and nocodasol, and did not correlate to actin or tubulin polymerization. According to Klymkowsky *et al.* (1987), cytokeratin filaments with the first and second cleavage form bundles like a loose fish net, which encompasses the vegetal portion in *Xenopus*. Amphibian eggs show total cleavage but fish eggs display discoidal cleavage. In fish, therefore, a firm cytokeratin network may form in the yolk body at the vegetal hemisphere during blastodisc formation.

The fusion of two fertilized eggs entails the existence of two cells with different genomes, since the two nuclei do not fuse together. The blastomeres from two fused eggs form a chimeric fish. Viable chimeric fish obtained from electric cell fusion contains several embryological problems, such as (1) embryonic axis determination, (2) sexual determination. Especially, it remains unclear whether fused eggs, in which each egg contains an entire body plan, develop into normal embryos with one embryonic axis. Now we are studying the spatial distribution of the determinants of dorso-ventral axis in fish egg. We will discuss this problem elsewhere.

From these results, other possibilities for electric cell fusion to denuded eggs are shown in this study except for nuclear transplantation, namely (1) introduction of certain materials into egg cytoplasm, (2) addition of exogenous nutrition to yolk body, and (3) production of viable chimeric fish (Fig. 7).

Hybridizations were attempted in many kinds of teleost fishes. When the reciprocal hybrids show different survival rates, it was thought to be because of incompatibilities between male genome and egg cytoplasm (Suzuki, 1968; Suzuki and Fukuda, 1971). These incompatibilities have not been proven directly. If cytoplasms from two species are mixed, the results will make clear the reasons. Certain kinds of hybrid sterility or lethality may depend on some kind of cytoplasmic factors. Introduction of cytoplasm from one species to another will be useful for screening of unknown cytoplasmic factors, concerned with male sterilization, for example.

Normal embryos developed from fused egg with one blastodisc. These double sized embryos developed and grew faster than those of normal size, because of their double sized yolk and large mouth size. Present results also suggest that additional exogenous nutrition derived from fusion of yolk body may cause a larger embryo

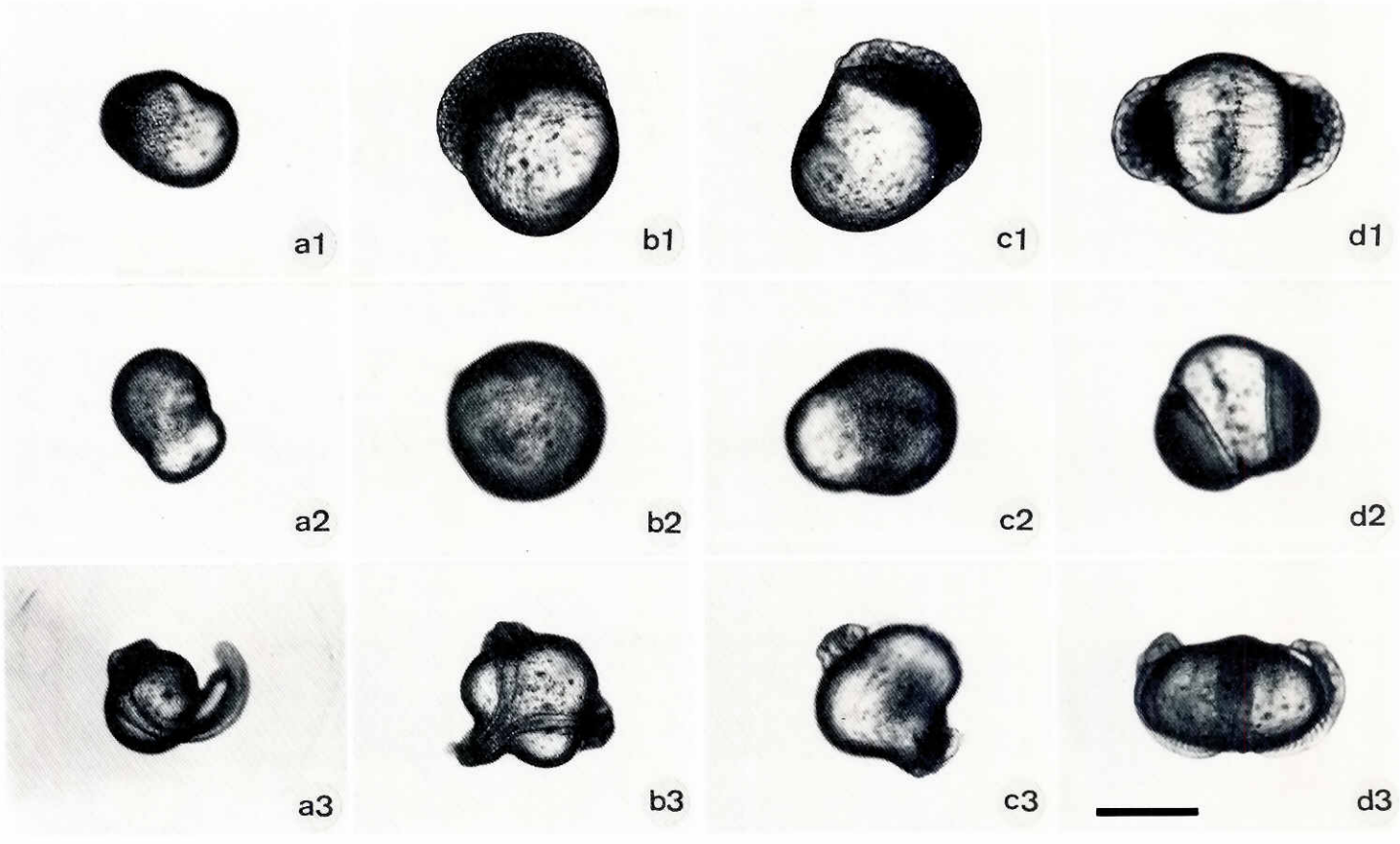

Fig. 4. Development of fused and control eggs. (a) *Control;* **(b and c)** *fused egg with one blastodisc;* **(d)** *fused egg with two blastodiscs.* **(Row 1)** *Blastula stage;* **(row 2)** *early gastrula stage;* **(row 3)** *36 h after fusion. Bar indicates 0.5 mm.*

than normal. For example, the egg of *Olyzias latipes* is the same size as that of goldfish, but the hatched larva is smaller. If the additional yolk of the same or other species, such as goldfish, is fused to *Olyzias* egg, it will be expected to increase the body size of fry and to feed them easily.

Chimeric animals are useful for investigating cell-to-cell interaction. In mice, several 4 to 8 cell embryos aggregated with phytohemagglutinin developed into one individual. This chimeric mouse was used for investigation of the nervous system, gonadal system or immune system (Kato and Mikoshiba, 1982). In the chimeric system, it is important to discriminate the cells from different embryos with genetical markers, such as number of nucleoli or ploidy in amphibians, morphological difference of nuclei in avians, and genomic sequences in mouse. There are natural triploid or tetraploid populations (2N=100, 3N=156, 4N=206) in *Carassius auratus* named crucian carp, which reproduce gynogenetically and all-female clone populations (Kobayashi *et al.*, 1970). Artificial triploids were also induced by hydrostatic pressure treatment at the second meiotic division (Yamaha and Onozato, 1985). These ploidies will be useful in cell identification and in sexual or immune system differentiation in the fused embryos. It is important to produce fused eggs constantly with a single embryonic body so as to use the chimeric embryos to study cell differentiation. For these purposes, it is necessary to make clear how the organization of embryonic axis is decided.

Materials and Methods

Fertilization and dechorionation

Adult goldfish, *Carassius auratus*, were reared in the Aquarium Center of the Faculty of Fisheries, Hokkaido University. Mature eggs were ovulated spontaneously or induced by hormonal injection of 10 unit gonadotropin (pvelogen: Sankyo Seiyaku Co. Ltd.) per gram body weight. Sperm from mature males were collected with microcapillary tubes. The fusion was usually applied to the intrastrain eggs to establish the appropriate fusion condition, but the interstrain egg fusion, which had pigmented or non-pigmented clear scales, was carried out in order to discriminate chimeric condition. Artificial insemination was performed by the dry method. The eggs were stripped on vinylidene chloride film and inseminated with the milt. Dechorionation procedures of the fertilized eggs were based on Yamaha *et al.* (1986). Inseminated eggs were activated in a plastic or glass dish with the activate solution containing 0.4% urea and 0.5% NaCl to weaken the egg adhesion for about 2 min. Activate solution was changed to the dechorionation solution containing 0.1% trypsin (DIFCO), 0.4% urea Ringer solution (128 mM NaCl, 2.8 mM KCl, 1.8 mM $CaCl_2$) with pH adjusted to 7 by NaOH, and eggs were treated with it for about 10 min. Dechorionated eggs were transferred to the Ringer's solution and used for each experiment.

Chemical fusion by polyethylene glycol

Polyethylene glycol 6000 (Nakarai Chemicals Ltd) was dissolved in Ringer's solution (128 mM NaCl, 2.8 mM KCl, 1.8 mM $CaCl_2$) at 50% W/V. Chemical fusion was attempted on the denuded eggs as follows:

i) Pour 50% polyethylene glycol 6000 just above the denuded eggs in Ringer's solution and treat for 10 minutes or longer.

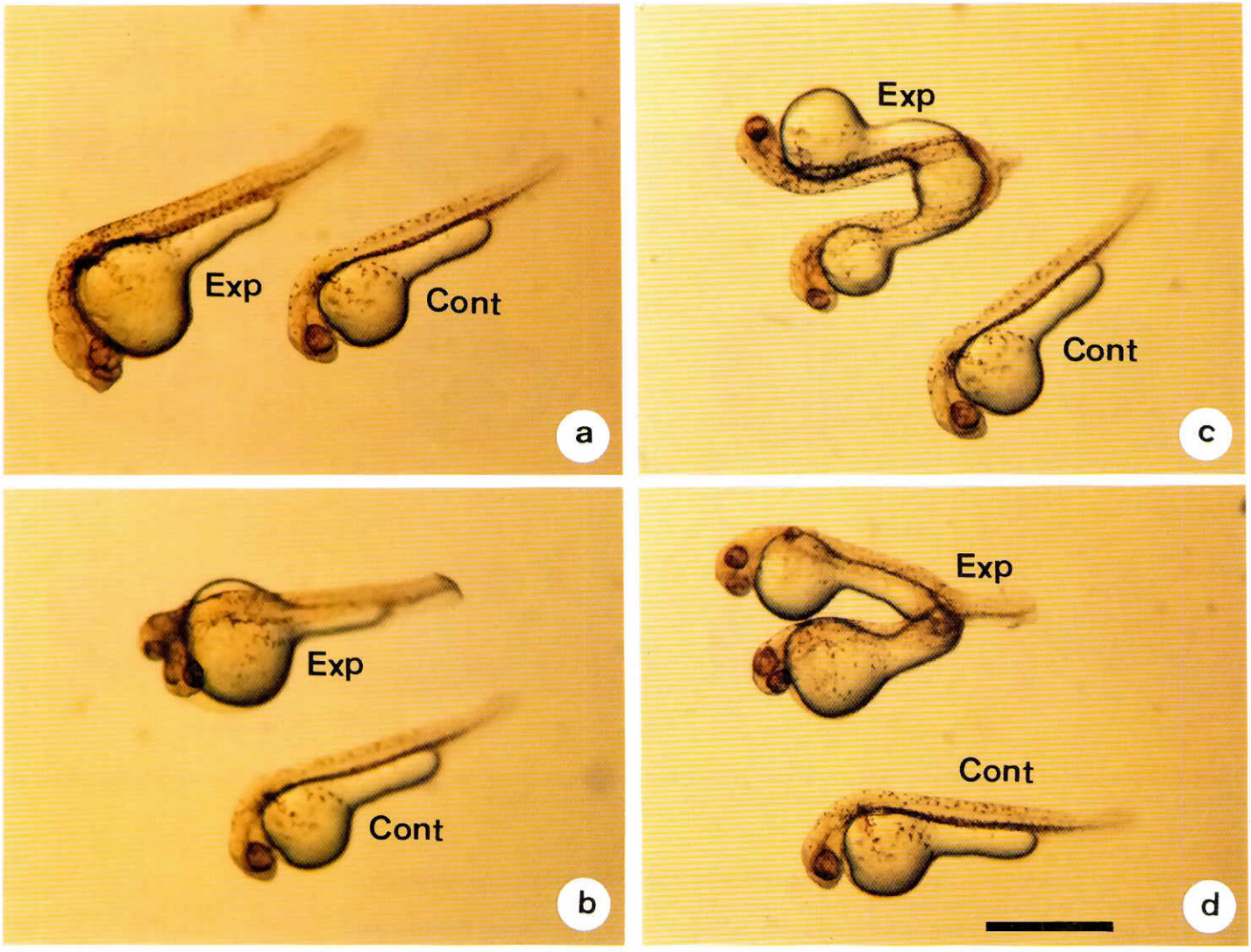

Fig. 5. Larvae from fused and control eggs at the hatching stage. (a,b) *Larvae from a fused egg with blastodisc and a control egg.* **(c,d)** *Larvae from a fused egg with two blastodiscs and a control egg. Exp, embryos from the fused egg; Cont, embryos from the denuded egg. Bar indicates 1 mm.*

ii) Transfer the adhered eggs to the Ringer's solution and wash them twice.

Electric egg fusion

Denuded fertilized eggs were fused successfully by electric cell fusion as per the following procedure (Fig. 1).

i) Inseminate and activate the eggs in the activate solution for 1 min in plastic dish at 20 °C.
ii) Change the activate solution to the dechorionation solution.
iii) Transfer the denuded eggs to the Ringer's solution 10 min after activation, and wash them briefly with 100 μM EDTA solution two times and then with 25 μM EDTA solution one time.
iv) Settle several pairs of the eggs side by side between the electrodes at 2 mm interval filled with 25 μM EDTA solution.
v) Apply to the eggs with 400V/cm, 1MHz dielectrophoresis pulses for 3 seconds and a successive 750 V/cm, 10 μsec fusion pulse by the electric fusion instrument SSH10 (Shimazu Seisakusho, Co. Ltd.)
vi) Pour gently with 1.8 mM $CaCl_2$ solution over the paired eggs just after application of fusion pulse and leave for about 30 minutes.
vii) Transfer the fused eggs to Ringer's solution and culture under the following conditions.

Cultivation of treated and control denuded eggs

Treated or control denuded eggs were cultured in all 96 wells of culture dishes (Falcon) filled with culture Ringer's solution containing 1.6% albumen, 0.01% penicillin, 0.01% streptomycin, 0.01% kanamycin for 1 day at 20°C. On the next day, eggs were moved to the wells of culture dishes filled with 1.8 mM $CaCl_2$, 1.8 mM $MgCl_2$ and appropriate antibiotics solution. The eggs were incubated at 20°C for 3 days, until the intact eggs hatched out. These larvae were moved to either the plastic dishes or the 24-well culture dishes filled with dechlorinated tap water, and then to bigger aquariums depending on the size of their body. They were fed with the larvae of *Artemia salina*, or with commercial food in powder for carp.

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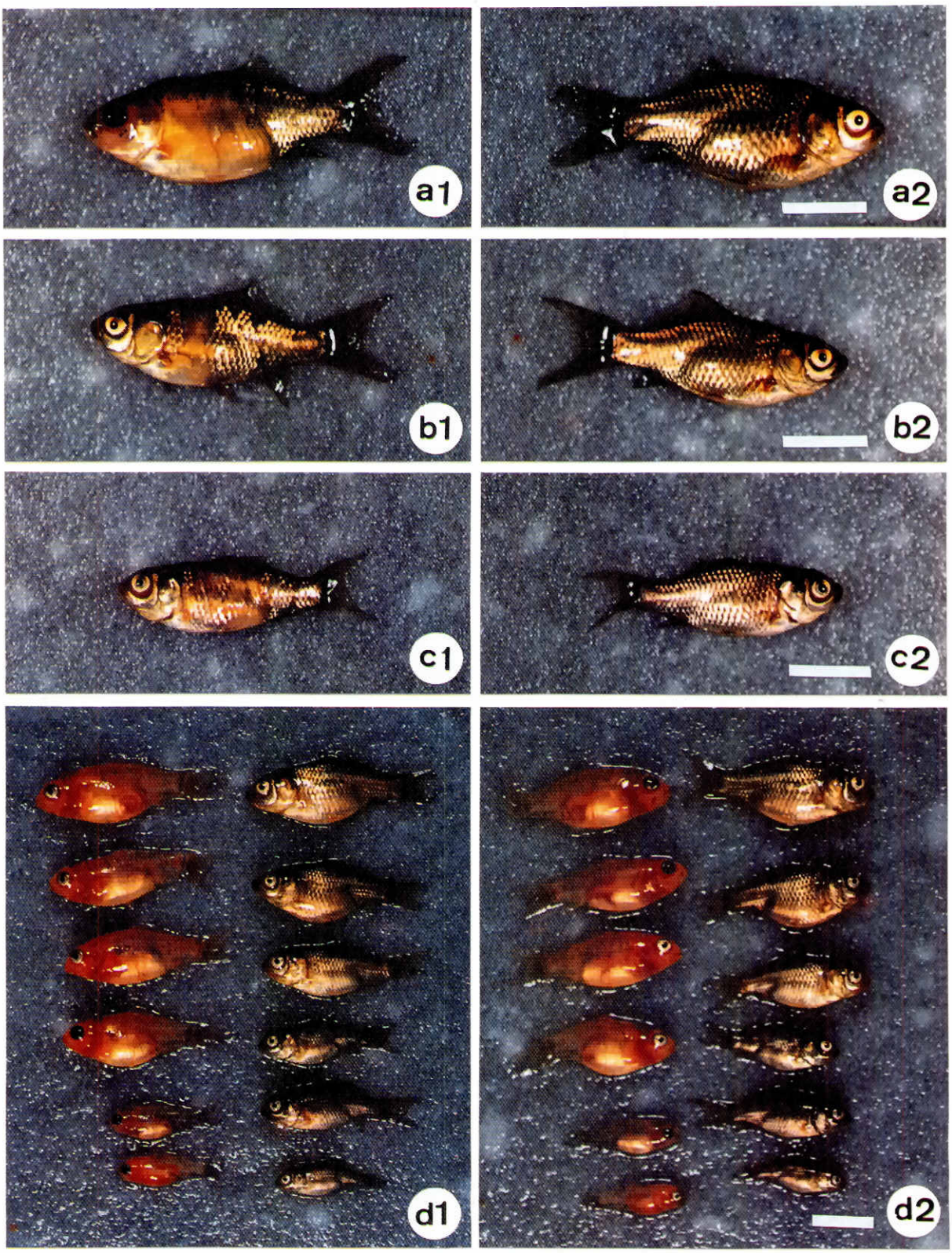

Fig. 6. Young fish from the fused and control eggs. (a-c) *Fish from fused egg.* **(d)** *Control fish.* **(Column 1)** *Left side of the fish;* **(column 2)** *right side of the fish. Note that the fish from fused eggs have clear and pigmented scales on their left side and that no control fish have both types of scales. Bar indicates 1 cm.*

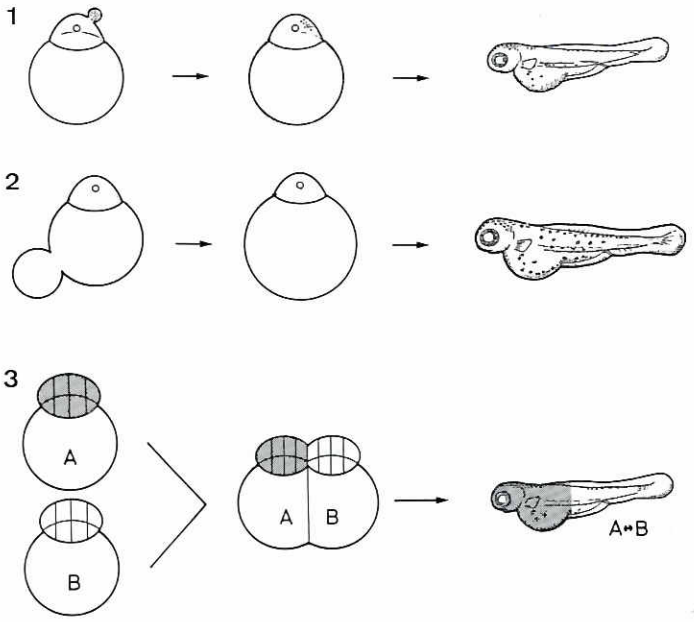

Fig. 7. The possibilities of cell fusion technique on denuded eggs. (1) *Introduction of some kinds of materials of egg cytoplasm.* **(2)** *Addition of exogenous nutritients to yolk body.* **(3)** *Production of chimeric fish.*

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