Fusion of Fish Spermatozoa with Polyethylene Glycol

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In chromosome manipulations of fish, duplication of genomes has been achieved generally by inhibiting the nuclear division. Recently, it has been also reported that genomes can be duplicated through fertilization with fused spermatozoa. In this study, fusion conditions of polyethylene glycol (PEG) and motilities of fused spermatozoa were examined by using rainbow trout and nigorobuna spermatozoa. A comparison between two molecular weights of PEG indicated that a molecular weight of 4000 was superior in fusion ability to that of 1000. Fusion rate [(total number of spermatozoa participating in fusion/total number of tested spermatozoa)×100] of rainbow trout spermatozoa was higher than that of nigorobuna. In a molecular weight of 4000, fusion rates of rainbow trout spermatozoa at concentrations of 30, 40, and 50% were 28.4, 40.2, and 44.4% respectively, while those of nigorobuna were 5.0, 18.6, and 34.8 respectively. When the diameter of the micropyle is taken into consideration, fused spermatozoa capable of entering an egg are restricted to those consisting of two spermatozoa. The maximum fusion rates between two spermatozoa in rainbow trout and nigorobuna were respectively 18.5% (concentration: 40%) and 15.9% (concentration: 50%). The duration of the motility of PEG-treated spermatozoa did not vary significantly depending on the three treatment concentrations, and compared with the control ones, the motion times of those of rainbow trout and nigorobuna were shortened by 2/3 and 1/2, respectively.

Over the last ten years, many attempts have been made to culture fish with increased added value by chromosome manipulation. As a result, triploids can be almost surely obtained by suppressing the second polar body extrusion of the egg following fertilization, while gynogenetic diploids can be produced by insemination with genetically inert spermatozoa, and subsequently inhibiting the second meiosis or first cleavage. On the other hand, several attempts have been made to create tetraploids by disruption of the first cleavage mitosis and to induce androgenetic diploids by destruction of the second meiotic chromosomes of unfertilized eggs and suppression of the first cleavage of the eggs after activation by normal spermatozoa. However, the latter techniques have not yet reached the stage of mass production of the target fish, and therefore further improvements in these techniques are needed.

As described above, duplication of genomes in fish through chromosome manipulations has always been achieved by inhibiting cell division (karyokinesis) with temperature or hydrostatic

pressure shocks. Recently, Ueda *et al.*¹⁾ suggested another possibility related to genome duplication. According to their report, rainbow trout spermatozoa were fused with polyethylene glycol (PEG), and the eggs were inseminated with these spermatozoa obtained to thereby produce triploid fish. From the analysis of the karyotype, it was shown that the triploids thus obtained consisted of two paternal genomes and one maternal genome. Next, Ueda *et al.*²⁾ adhered or fused rainbow trout spermatozoa with the use of calcium-rich/high pH BSS. Here it was shown that triploid fish could be similarly obtained using these spermatozoa. It is expected that these methods with the use of fused or adhered spermatozoa might be applicable not only for the induction of triploids but also for the production of tetraploids and androgenetic diploids which can be hardly be produced by existing techniques.

Under these circumstances, the aim of this study is to solve the various problems related to the formation of fused spermatozoa with PEG. The first subject of this study centers around an ex-

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amination of the ability to fuse spermatozoa. On this point, the PEG concentration at which fusion between two spermatozoa occurs at the highest rate is discussed. The second subject of this study deals with the problem regarding the motility of PEG-treated spermatozoa. This problem is discussed by morphologically observing the fusion state and duration of the motility.

Materials and Methods

Collection of Sperm

Sperm from farmed rainbow trout (*Oncorhynchus mykiss*) and nigorobuna (*Carassius auratus grandoculis*, cyprinid fish) were used. The semen, which was collected by pressing the abdomen of the fishes, was placed in a watch glass. A small amount of the each semen sample was mixed with water on a slide glass and observed under a light microscope to examine sperm motility. Thus, those showing high motility were used for the test. Table 1 shows the detailed characteristics of the fishes employed in the test and the spermatozoon amounts of their semen.

Fusion of Spermatozoa

PEGs of 1000 and 4000 in molecular weight (Merck) were used and their concentrations were adjusted 30, 40, and 50% with a medium*³ having the same ion compositions as that of the seminal plasma of the corresponding fishes. Fusion of spermatozoa was carried out in the following manner in every test reported herein. 1) The collected sperm sample was diluted 1000-fold with the medium. 2) 10 ml of the diluted sperm was introduced into a 50 ml round-bottle centrifugation tube and centrifuged at 1500 rpm for 10 min. 3) After discarding the supernatant, the surface of the sperm pellet thus was coated with a thin layer of a 1 ml PEG solution, and then allowed to stand for 10 sec. 4) After adding 9 ml of the

medium, the mixture was gently shaken and the PEG solution was diluted, followed by centrifugation at 1500 rpm for 5 min. 5) The supernatant was removed and replaced with 10 ml of the medium. 6) Procedure 5) above, accompanied by centrifugation, was repeated twice. 7) After removing the supernatant and adding 5 ml of medium, the treated spermatozoa were suspended with the use of a pipette. However, control spermatozoa were treated in the same manner, except for the PEG-treatment [procedures 3) and 4)].

Examination of the Fusion Rate and Morphological Observation of Fused Spermatozoa

The suspension of the PEG-treated spermatozoa was placed on a Thoma hemocytometer, then unfused spermatozoa and fused spermatozoa (discriminating fusion between two spermatozoa and fusion between three or more spermatozoa) were counted. The fusion rate was expressed as the percentage of the spermatozoa participating in the fusion to the total (520–1200 spermatozoa) counted. Scanning electron microscope preparations were made in the following manner. Spermatozoa were placed on a cover glass, the surface of which was coated with protamine sulfate. After drying, the spermatozoa were fixed with 1% glutaraldehyde, dehydrated with an alcohol series, and then dried. Next, the sample was metallized with gold using a conventional method.

Examination of Motility of PEG-treated Spermatozoa

The suspension of PEG-treated spermatozoa and that of the control spermatozoa were each placed on a microscope slide. After diluting 10-fold with water (in the case of nigorobuna) or an egg rinsing solution (150 mM NaCl, 32 mM KCl and 23 mM CaCl₂, in the case of rainbow trout), the time required until all spermatozoa completely stopped moving was measured.

Table 1. Specification of the male fish used for sperm taking and sperm count

Fish	Age	No of individual	Body length (cm) range (mean)	Body weight (g) range (mean)	No of spermatozoa per 1 ml milt ($\times 10^3$) range (mean $\pm$ SD)
Rainbow trout	3 years	7	41.1–48.0 (43.9)	1000–1440 (1310)	129.0–206.2 (157.1 $\pm$ 29.4)
Nigorobuna	3 years	5	11.2–13.1 (12.4)	45–63 (51)	230.6–283.0 (240.9 $\pm$ 23.0)

*³ Medium for rainbow trout: 130 mM NaCl, 40 mM KCl, 2.5 mM CaCl₂, 1.5 mM MgSO₄, 10 mM HEPES, pH 8.2. Medium for nigorobuna: 96 mM NaCl, 70 mM KCl, 2 mM CaCl₂, 1 mM MgSO₄, 10 mM HEPES, pH 8.2.

Fig. 1. Comparison of sperm fusion rates between two molecular weights of PEG.

The values are the average of two or three measurements.

Δ: molecular weight 1000, ○: molecular weight 4000.

Results

Effect of PEG on Sperm Fusion

As shown in Fig. 1, the fusability of PEG at a molecular weight of 4000 was higher than that at a molecular weight of 1000, and the fusability was elevated with an increase in the PEG concentration. However, the fusion rate of *nigrolobuna* was lower than that of rainbow trout at every PEG concentration. At a molecular weight of 1000, *nigrolobuna* showed a fusion effect exclusively at a concentration of 50%. In the control, fused spermatozoa were observed at a rate of several percent. Although some of these spermatozoa might actually have been fused there was a great possibility that overlapping or contacting spermatozoa were miscounted as fused ones.

Fig. 2 shows the fusion rate of spermatozoa fused with two and those fused with three or more in two species. In rainbow trout, the fusion rate between two spermatozoa was not increased in correlation to PEG concentration and was lower than those of three or more spermatozoa at a concentration of 50% (molecular weight: 1000) or 40% (molecular weight: 4000). The same phenomenon was observed at a concentration of 50% in the case of a molecular weight of 4000 in *nigrolobuna*.

Fig. 2. Comparison of sperm fusion rates between spermatozoa fused by two and those fused by three or more.

The values are the average of two or three measurements.

○: fusion between two spermatozoa with a molecular weight of 1000, Δ: fusion between three or more spermatozoa with a molecular weight of 1000, ●: fusion between two spermatozoa with a molecular weight of 4000, ▲: fusion between three or more spermatozoa with a molecular weight of 4000.

Effect of PEG Treatment on Motility of Spermatozoa

As shown in Fig. 3, the maximum duration of sperm motility of the control rainbow trout spermatozoa was approximately 45 sec, while the time of the treated spermatozoa varied very little depending on molecular weight and remained around 30 sec for each concentration. The duration of motility in control *nigrolobuna* spermatozoa was approximately 250 sec, while treated spermatozoa showed a time of 150 sec or less at each molecular weight. Further, the motility tended to decrease as the concentration increased.

Morphology of Fused Spermatozoa

Except for the deletion of the tail in some spermatozoa, the PEG-treatment caused no structural damage to spermatozoa. Fusion occurred at every part of the spermatozoa, namely, head, mid-piece, and tail. When fusion occurred between heads, heterocaryon as commonly observed in cell fusion was not formed but partial fusion between plasma membranes covering the head was ob-

Fig. 3. Effect of PEG treatment on sperm motility.

The values are the average of two or three measurements. Vertical lines represent the range.

Δ: molecular weight 1000, ○: molecular weight 4000.

served (Fig. 4). This is because spermatozoon heads almost exclusively consist of nuclei and contain few cytoplasmic factors. When spermatozoa were fused with each other in such a manner that their heads were in the same direction (Fig. 4), the fused spermatozoa could move forwards. The rate of the movement varied depending on the condition of the fusion between the tails.

Discussion

PEG Concentration Suitable for Fusion of Spermatozoa

PEG of a molecular weight of 4000 was suitable for the fusion of rainbow trout and nigorobuna spermatozoa. Although an increase in PEG concentration caused an increase in the fusion rate, it induced the formation of a number of aggregates of spermatozoa and, as a result, the fusion rate between two spermatozoa was lowered. In a molecular weight of 4000, the suitable concentrations for fusion between two spermatozoa of rainbow trout and nigorobuna are apparently 40 and 50%, respectively. At these PEG concentrations, slightly less than 20% of spermatozoa participated in the fusion between two spermatozoa. Although this fusion rate is not so high, the semen of these fishes contain spermatozoa which ensure the production of fused spermatozoa in an amount sufficient for successful fertilization (Table 1). The PEG treatment scarcely damaged the basic structure of spermatozoa. This fact suggests that fusion spermatozoa would exert their normal function after entering into the eggs. The moving time of fused spermatozoa was shortened by 2/3 (in the case of rainbow trout) or 1/2 (in the case of nigorobuna), each compared with the control ones. In common fertilization, however, spermatozoa would penetrate the eggs instantly. Thus, it seems that shortening the fused spermatozoa motility, as discussed above, might cause no problem in fertilization.

Application of Fused Spermatozoa to Chromosome Manipulation

Triploidy in shellfish has been induced by inhibiting a release of the first or second polar body. A study on polyploidy induction of American oysters has revealed that triploids in oysters created during the first meiosis show a higher growth than those created during the second meiosis, due to greater heterozygosity.²⁰ Triploid fish have hitherto been produced only by inhibition of the second meiosis. In this case, the effects of heterosis do not promise much. This disadvantage can be avoided by inducing triploids with the use of fused spermatozoa. It is ensured that the formation of triploidy enables duplication of paternal genetic material, which differs from that of the female genome employed hitherto, and thus enlarges the application range of autotriploids and allotriploids during breeding.

Fig. 4. Scanning electron micrographs of fused spermatozoa after PEG treatment.

A: fusion between two nigorobuna spermatozoa, B and C: fusion between three or more rainbow trout spermatozoa, D: aggregate consisting of a number of rainbow trout spermatozoa.

To induce androgenetic fish, the second meiotic metaphase chromosomes of eggs should be inactivated prior to insemination by gamma rays⁴⁻⁶⁾ or ultraviolet radiation.⁷⁾ However, methods using radiation are not always the most suitable. This is because the high permeability of radiation might not only damage chromosomal DNA but also exert undesirable effects on all substances which are necessary in the early stage of the egg development. On the other hand, radiation with ultraviolet of a low permeability makes it necessary to accurately irradiate mitotic apparatus, but the location of these spindles in the fish eggs, most of which are spherical, can hardly be judged from appearance. To obtain survivable androgenetic diploids, the first division should be subsequently inhibited by, for example, hydrostatic pressure. These physical treatments should significantly prevent egg development. Although the function of spermatozoa is completed in fertilization, the eggs should continuously maintain their life formed by fertilization. This fact suggests that it is suitable to subject not eggs but spermatozoa to the induction treatment of androgenetic diploids.

It has been reported that the creation of tetraploid fish at high frequency is very difficult.^{8,9)} It is expected that tetraploids may be obtained by inseminating the eggs with spermatozoa fused by two and inhibiting the second meiosis. Furthermore, it is expected that tetraploids might be

obtained by fusing UV-treated spermatozoa with a somatic cell of the late S phase or thereafter, and then inseminating the eggs with the spermatozoa thus obtained. The process of sperm fusion would contribute to the technical development of chromosome manipulations through a path different from existing ones.

Problems in Utilization of Fused Spermatozoa

Micropyle consists of a vestibule and a micropylar canal. Electron microscopic observation has revealed that the diameter of the micropylar canal is large enough to allow only one (chum salmon)¹⁰⁾ or two (medaka and carp)^{11,12)} spermatozoa to pass through it in parallel. Although it might not be appropriate to judge the capacity of the invasion of fused spermatozoa into the eggs based on these results of the observation of fixed preparations, the above-mentioned facts suggest that chromosome manipulation with the use of fused spermatozoa is not applicable to all fish. On the other hand, it is also suggested that fused spermatozoa consisting of three or more spermatozoa are thus selected at the micropyle, and thus, never undergo fertilization. In this technique, it is extremely important to develop a method for distinguishing a single spermatozoon from fused ones consisting of two spermatozoa.

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