On the Subzero Temperature Preservation of Fertilized Eggs of Rainbow Trout

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(Received May 4, 1982)

A possibility of preservation of fertilized eggs of rainbow trout *Salmo gairdneri* was examined at $-7^{\circ}\text{C} \sim -20^{\circ}\text{C}$ for 3 hours. The results obtained are summarized as follows;

1. Eggs after eyed stage had higher tolerance for subzero temperature exposure than those of before eyed stage.
2. Suitable concentration of dimethyl sulfoxide (DMSO) as organic cryoprotectant was 1.4~2.1 M.
3. In -7°C exposure, about 95% of hatching rate was obtained by using 10% calf serum and mineral salts solution besides DMSO as cryoprotectants, while 14% of hatching rate was obtained in -12°C exposure.

The cryopreservation of fish sperm has been investigated by many authors.¹⁻¹⁰⁾ Scientific reports on the subzero temperature preservation of fish eggs are, however, comparatively few. WHITTINGHAM & ROSENTHAL¹¹⁾ reported about subzero temperature preservation of fertilized eggs of Atlantic herring. But there is no report concerning the subzero temperature preservation of fertilized eggs of freshwater fish.

This report deals with a possibility of subzero temperature preservation of fertilized eggs of rainbow trout as a model of cryopreservation of fertilized eggs of valuable cultured freshwater fish.

Materials and Methods

Eggs used were hand-stripped from the matured female rainbow trout obtained from fish culturist. The eggs were artificially fertilized by isotonic method after rinsing eggs with physiological saline.¹²⁾ They were incubated at *ca.* 12°C in Atkins style incubators until used in the experiments. And temperature units were found from the daily mean value of water temperature.

Through the experiments, eggs tested were placed in several aquatic media consist of mineral salts and organic cryoprotectants. The mineral salts solution was composed of 3.2 mM KCl, 2.3 mM $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ and 154,171 and 188 mM NaCl. DMSO, glucose and calf serum were added to mineral salts solutions as cryoprotectants.

Thirty fertilized eggs were placed in a test tube (22 mm in diameter) having 20 ml of a medium previously cooled to $4.0 \sim 7.2^{\circ}\text{C}$. Then, the test tube containing eggs and medium was placed in a cooling bath and cooled at the rate of $0.4^{\circ} \sim 0.9^{\circ}\text{C}/\text{min}$ until -5°C and at $0.7^{\circ} \sim 8.9^{\circ}\text{C}/\text{h}$ below -5°C . After the test tubes were exposed to the constant subzero temperature for 3 hours, they were taken out from the cooling bath and kept in a water tank filled with *ca.* 12°C water for rising the eggs' temperature. The eggs warmed to 12°C were placed on incubator trays, and washed several times by water, then they were replaced into the incubator to observe whether or not hatching had occurred. Hatching rates were shown as the indices comparing with the hatching numbers of untreated eggs.

Eggs were carefully handled with tweezers made of feathers of water bird. The temperature was monitored by a thermojunction thermometer.

Results and Discussion

Developmental Stage and Subzero Temperature Tolerance

Five different stages of eggs were examined to investigate the suitable stage regarding subzero temperature tolerance. As shown in Table 1, the eggs until 6 days after fertilization stage did not hatch, but the eggs after eyed stage hatched. Hatching rate increased gradually from before eyed stage to before hatching stage. The eggs before hatching

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Table 1. Developmental stage and hatching rate.

Eggs were exposed to -8°C . Medium used was composed of 1.4 M DMSO, 3.2 mM KCl, 2.3 mM $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ and 171 mM NaCl

Developmental stage	Hatching rate (%)
Just after fertilization (0)	0
6 days after fertilization (82)	0
Before eyed stage (171)	5
Eyed stage (207)	30
Before hatching (289)	50

() : total effective temperature ($^{\circ}\text{C}$).

stage showed maximum hatching rate.

It was reported that eyed eggs of salmonid fish had high tolerance against physical stimulus.¹⁸⁻¹⁹ The same phenomenon was observed in this experiment, namely, the eyed eggs showed higher tolerance in the subzero temperature exposure. From above results, eyed eggs (total effective temperature: about 300°C) were used in the following experiments.

Effect of DMSO Concentration

Although DMSO has been known to be a useful cryoprotectant in cryopreservation of mammalian embryos and fish sperm, there is only one report concerning the cryopreservation of fertilized fish eggs (Atlantic herring).¹¹ Therefore, DMSO was examined whether it is useful to cryopreservation of fertilized eggs of rainbow trout.

As shown in Fig. 1, in -7°C exposure any of the eggs tested never hatched without DMSO addi-

Fig. 1. Effect of DMSO concentration on hatching rate. Eggs were exposed to -7°C with two media prepared as mentioned below. DMSO was added to the mineral salts solution consists of 3.2 mM KCl, 2.3 mM $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ and NaCl. ●—●: 154 mM NaCl. ○—○: 188 mM NaCl.

Fig. 2. Effect of glucose addition on hatching rate. Eggs were exposed to -7°C with two media prepared as mentioned below. Glucose and 2.1 M DMSO were added to the mineral salts solution consists of 3.2 mM KCl, 2.3 mM $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ and NaCl. ●—●: 154 mM NaCl. ○—○: 171 mM NaCl.

tion, but ca. 40% of hatching rate was obtained by 1.4~2.1 M of DMSO addition. Remarkable difference in the hatching rate was not recognized between 154 and 188 mM of NaCl concentration. In -10°C exposure, hatching did not occur in spite of 0.7~4.2 M of DMSO addition.

These results indicate that the addition of 1.4~2.1 M of DMSO at -7°C is one of the useful cryoprotectants for the cryopreservation of fertilized eggs of rainbow trout.

Effect of Glucose Addition

By using a medium containing DMSO and mineral salts, maximum of 47% of hatching rate was obtained (Fig. 1). Therefore, additional effect of glucose to the medium containing DMSO and mineral salts was examined in order to increase hatching rate.

As shown in Fig. 2, 11.1 mM of glucose addition raised hatching rate to ca. 95% in -7°C exposure. In -10°C exposure, hatching did not occur in spite of 5.6 or 11.1 mM of glucose addition.

These results indicate that the addition of glucose to the medium is advantageous over the medium without glucose for cryopreservation of eggs of rainbow trout.

Effect of Calf Serum Addition

As shown in Fig. 3, the addition of 10~20% of calf serum to the medium containing DMSO and mineral salts in -7°C exposure showed same

Fig. 3. Effect of calf serum addition on hatching rate. Eggs were exposed to -7°C with two media prepared as mentioned below. Calf serum and 2.1 M DMSO were added to the mineral salts solution consists of 3.2 mM KCl, 2.3 mM $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ and NaCl. ●—●: 154 mM NaCl. ○---○: 171 mM NaCl.

Fig. 4. Effect of subzero temperature exposures by addition of calf serum on hatching rate.

Ten per cent of calf serum was added besides DMSO (1.4 M) to the mineral salts solution consists of 3.2 mM KCl, 2.3 mM $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ and 171 mM NaCl.

effect (hatching rate of ca. 95%) as in the addition of 11.1 mM of glucose. Moreover, temperature at which eggs survived was lowered to -12°C by the addition of 10% of calf serum as shown in Fig. 4.

These results indicate that calf serum is more useful than glucose as cryoprotectant for cryopreservation of eggs of rainbow trout.

In -12°C exposure, a part of hatched larvae showed abnormal physiological phenomena, i.e.,

stopped breath, internal hemorrhage in head and opaquely white caudal part. These abnormal larvae died ca. 3 days after hatching. At -7°C or -10°C in the high concentrations of DMSO (above 2.1 M), many of media used were not frozen. On the other hand, in the low concentrations of DMSO or at lower temperature (-12°C or -20°C), all media used were frozen. It was reported previously that the freezing of egg's intracellular substances was fatal to the unfertilized eggs of Chum salmon,¹⁷⁾ and the free-intracellular water was dehydrated with DMSO, and hence -197°C exposure was not fatal to rat embryos.¹⁹⁾ Serum was contained in the medium of cryopreservation of mouse embryos^{18,20)} and rat embryos,¹⁸⁾ but there was no description about the effect of the serum on cryopreservation.

From the results of this study, high concentration of DMSO showed a hindrance to hatching as shown in Fig. 1 and the necessity of adding calf serum was demonstrated as additional cryoprotectant for subzero temperature exposure.

Acknowledgements

The author expresses his gratitude to Dr. K. KODAMA, Central Research Institute of Electric Power Industry (CRIEPI), for constant guidance in the course of the work, and to Mr. H. HONDA, CRIEPI, for valuable advice.

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