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# Cryosurvival of goldfish embryo after subzero freezing

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**Abstract**

Goldfish embryos of pre-morula, somite formation, optic vesicle, heart formation and pigment formation stages were tested for cryoprotectant toxicity, using DMSO and glycerol at 10, 12 and 15% concentration. Those that showed 50% or more survival were employed for cold shock test, from 24 to 4°C at the rate of −1°C/min. Only embryos at the heart formation stage protected with 12% DMSO showed 60% survival and 35% hatching. A group of embryos of that stage went through a freezing and thawing test to temperatures of −15, −25 and −35°C. They showed 30, 5 and 2% survival and 12, 3 and 1% hatching respectively.

**Keywords:** Goldfish, *Carassius auratus*, Cyprinidae, embryo, cryosurvival.

*Survie des embryons de poisson rouge après conservation à une température voisine de 0°C.*

**Résumé**

La toxicité des cryoprotecteurs, le DMSO et le glycérol, à 10, 12 et 15 % ont été testés sur des embryons de poisson rouge, aux stades de développement suivants: pré-morula, formation des somites, vésicule optique, formation du cœur, et pigmentation. Le taux de survie est de 50 % pour des tests de chocs thermiques froids, de 24 à 4°C, au régime de −1°C par minute. Seuls, les embryons au stade de formation cardiaque, cryoconservé au moyen du DMSO à 12 % présentèrent 60 % et 35 % d'éclosion. Des groupes d'embryons de ce stade résistèrent à des tests de températures de congélation, de −15, −25 et −35°C. Leur taux de survie a été de 30, 5 et 2 %, et leur taux d'éclosion de 12, 3 et 1 % respectivement.

**Mots-clés :** *Carassius auratus*, Cyprinidé, cryoconservation, embryon, cryoprotecteur.

## INTRODUCTION

The methodologies, development and application of cryopreservation of fish spermatozoa and ova from 1853 to 1976 was reviewed and well discussed by Horton and Ott in 1976. Based on the technical advances in cryobiology and success in cryopreservation of gametes of fish an international gametes bank was proposed for a wide range of applications (Harvey, 1987). At first, most cases of successfully cryopreserved fish gametes were spermatozoa. In the past 10 years, some information on the cryopreservation of fish eggs and embryos, with limited success, was reported some for species: carp (Zhang *et al.*, 1987), multicolour fin rainbow fish, olive flounder, red sea bream (Sasaki *et al.*, 1988) rainbow trout (Stoss and Donaldson, 1983) and other salmonids (Harvey, 1982). In general the survival rate after cryopreservation of embryos was far lower than that of sperm. The size of the embryos, which with a large amount of yolk are larger than sperm, and a more complicated

structure as they develop could be the major reasons for difficulty of preservation. Besides, other factor such as different developmental stages must be considered in order to obtain a consistent and manageable working procedure. This communication presents the preliminary results from the study of goldfish embryos (*Carassius auratus*) including cryoprotectant toxicity, cold shock test and survival at different subzero temperature ranges.

## MATERIAL AND METHODS

Mature, 2 to 3-year-old goldfish obtained from a private commercial hatchery were brought into the laboratory to prepare for spawning induction. They were put into a holding tank, of 2000 litres capacity, for a week to adapt to the new environment.

### Procedure for spwaning induction

At the time for spawning induction, the tank water was replaced by 1/3 to 1/2 with fresh water. Water plants, *Pistia stratiotes* and *Eichhornia crassipes*, with hair roots were floating on the surface. The tank was sprinkled with a shower installation. Each time, about a dozen goldfish were employed, and the male to female ratio was adjusted to 3 to 1. Under this arrangement the male started to chase the female within a day. After the active chasing, eggs were laid the next morning, and were ready for harvesting from the hairy rootlets of the water plants. At temperature at about the around 25°C, the embryos harvested from hairy roots were 4-cell stage. At the same temperature the eggs hatched within 72 hours and at 18 to 20°C the hatching time was around 96 hours. The development stage of embryos employed in both cryoprotectant toxicity and cold shock tests was controlled both by water temperature and time required for developing to a certain stage under close observation with a dissecting microscope.

### Cryoprotectant toxicity test

Harvested, fertilized, goldfish eggs, were kept in small watch glasses with tank water for further development. Having reached each of the following stages: pre-morula, somite formation, optic vesicle, heart formation and pigment formation, they were separated for cryoprotectant toxicity test.

In the toxicity test, two kinds of cryoprotectants, DMSO and glycerol, at three final concentrations of 10, 12 and 15% were used.

All embryos , for all three tested concentrations of both cryoprotectants, were started from a concentration of 5% and then moved to 8, 10, 12 and 15%, and stayed in each concentration for 10 min consecutively. After the 10% cryoprotectant treatment a group of embryos received no further treatment and was checked for survival. The other two groups of embryos were changed to 12% cryoprotectant for another 10 min. One group of these embryos was changed to 15% cryoprotectant for another 10 min for the highest concentration of the cryoprotectant toxicity test. Another group of embryos was kept without cryoprotectant as control.

At each concentration, the test was repeated 3 to 5 times. In each test 10 to 15 embryos were used depending on the number of embryos harvested. To change to higher concentration a dropper or syringe was used to take out and put in cryoprotectant, and the embryos were kept in the same container all the time.

### Cold shock test

The developmental stages of embryos which showed over 50% survival in the toxicity test were selected for the cold shock test. The rate of temperature decrease was 1°C/min from 24 to 4°C. The temperature decrease was achieved in a refrigerator. The final temperature reached was that measured of the water close to the embryos. After the final temperature was reached the embryos were put in tank water at 24°C for further development. The survival rate after cold shock test was based on the number of embryos that continued to develop after they had been put in tank water, and the hatching rate was based on the number of embryos hatched to fry.

### Freeze-thaw test

As a result of toxicity and cold shock tests, 12% DMSO was selected as the cryoprotectant concentration and the embryos of heart formation stage were selected for the freeze-thaw survival test. Embryos with similar development (heart formation) were equilibrated with DMSO started at 5% and continued through 8, 10 and to the final concentration, 12%. The duration in each concentration was 10 min. After cryoprotectant treatment the embryos were checked for their development, and the surviving ones were put into the same refrigerator to undergo the same rate of temperature decrease as used in the cold shock test. After the temperature of the water had dropped to 4°C at the rate of −1°C/min the embryos were quickly transfered into the freezer and the freezing rate was set at −0.35°C/min to the final temperatures, −15, −25 and −35°C respectively. Once the final temperature had been reached, the embryos were kept at that temperature for 60 min; then the watch glasses were removed from the freezing apparatus and quickly dropped into 24°C tank water for thawing. Then the embryos were checked for survival and their hatching rate. The criterion of after-thawing survival was immediate after-thawing heart

beating and the hatching rate was based on the number of hatched fry. The survivals data from the toxicity test without freezing were used as control reference for the freeze-thaw test.

In cold shock and freezing tests, refrigerator, deep freezer, and liquid nitrogen purchased locally were used. For temperature monitoring a Honeywell thermorecorder was used.

## RESULTS

In the cryoprotectant toxicity test (*table* 1), the embryos before morula stage in 10% DMSO or glycerol showed 55% survival as compared with control of 85% survival. The survival rates dropped as the concentration of both cryoprotectants increased from 10 to 12 and then 15%. At somite formation stage only 10% DMSO allowed 50% survival. At optic vesicle stage 10 and 12% DMSO gave higher survival rates (81 and 75% respectively). All the concentrations of glycerol and 15% DMSO gave less than 34% survival. At heart formation stage, all three concentrations of glycerol and 15% DMSO gave less than 26% survival, whereas the control resulted in 95% survival. The 10 and 12% DMSO treatment gave 85 and 80% survival respectively. At pigment formation stage, all three concentrations of glycerol and 15% DMSO gave lower than 35% survival as compared with 92% survival of control. In the toxicity test, treatment with three different DMSO and glycerol concentrations showed that the higher the concentration the lower the survival rate. The samples treated with 12% DMSO showed a 5 to 15% drop in survival from that of the 10% treated samples. The 15% DMSO treated samples showed survival rates 50 to 20% less than those in the 12% DMSO treated samples. The glycerol treatment had a similar effect with the three concentrations.

Among the samples that showed over 50% survival in the toxicity test only the embryos at heart formation

**Table 1.** – Toxicity test of cryoprotectants DMSO and glycerol, with three different concentrations, 10, 12 and 15%, and different developing stages of goldfish embryos.

| Developing stages | Cryo-protectant | Concen-tration (%) | Survival* (%) |
|---|---|---|---|
| Pre-morula | DMSO | 10 | 55 |
| | | 12 | 40 |
| | | 15 | 25 |
| | Glycerol | 10 | 56 |
| | | 12 | 34 |
| | | 15 | 21 |
| | control | – | 85 |
| Somite formation | DMSO | 10 | 50 |
| | | 12 | 45 |
| | | 15 | 15 |
| | Glycerol | 10 | 30 |
| | | 12 | 24 |
| | | 15 | 8 |
| | control | – | 75 |
| Optic vesicle | DMSO | 10 | 81 |
| | | 12 | 75 |
| | | 15 | 22 |
| | Glycerol | 10 | 33 |
| | | 12 | 28 |
| | | 15 | 5 |
| | control | – | 90 |
| Heart formation | DMSO | 10 | 85 |
| | | 12 | 80 |
| | | 15 | 15 |
| | Glycerol | 10 | 25 |
| | | 12 | 15 |
| | | 15 | 5 |
| | control | – | 95 |
| Pigment formation | DMSO | 10 | 82 |
| | | 12 | 75 |
| | | 15 | 35 |
| | Glycerol | 10 | 30 |
| | | 12 | 20 |
| | | 15 | 15 |
| | control | – | 92 |

* Continuing development.

**Table 2.** – Cold shock test of the goldfish embryos of different developing stages from over 50% survival in cryoprotectant toxicity test.

| Development stage | Cryo-protectant treatment | Survival rate in cryo-protectant (%) | Cold shock survival rate (%) | Hatching rate (%) |
|---|---|---|---|---|
| Pre-morula | DMSO 10% | 55 | 0 | – |
| | Glycerol 10% | 56 | 0 | – |
| Somite formation | DMSO 10% | 50 | 0 | |
| Optic vesicle | DMSO 10% | 81 | 0 | – |
| | 12% | 75 | 12 | 0 |
| Heart formation | DMSO 10% | 85 | 0 | |
| | 12% | 80 | 65 | 35 |
| Pigment formation | DMSO 10% | 82 | 0 | – |
| | 12% | 75 | 0 | |

**Table 3.** – Cryosurvival of goldfish embryos of heart formation stage.

| Temperature reached | Survival (after-thawing heart beating) (%) | Hatching (%) |
|---|---|---|
| −15°C | 30 | 12 |
| −25°C | 5 | 3 |
| −35°C | 2 | 1 |

Both survival and hatching percentage were compared with those that only went through cold shock test.

stage of the 12% DMSO treated group gave consistent survival and hatching rate in the cold shock test. The other stages combined with cryoprotectant treatments showed either no survival at all or occasionally showed a few survivals but no hatching (*table* 2).

In the freeze-thaw test, the lowest temperature (−35°C) gave a survival rate of 2 and hatching of 1%. The survival for −15°C was 30 and hatching 12%. The survival for 25°C was 5 and hatching 3%. As a general rule, as the final temperature decreased so did both the survival and the hatching rates (*table* 3).

## DISCUSSION

All the stages showed low survival in the toxicity test and none passed the cold shock test completely. Only the heart formation stage showed some survival and hatching. The survival rates decreased as the final temperature was lowered. Using the same cryoprotectant, DMSO, and similar freezing rate, 0.3-0.35°C/min, frozen to −10, 25 and −30°C, coho salmon (*Oncorhynchus kisutch*) eggs showed 25.3% after-thawing development rate after −10°C, but after neither −30°C nor −20°C did they show any after-thawing survival at all (Stoss and Refstie, 1983). Also using DMSO as cryoprotectant, zebra fish (*Brachydanio rerio*) eggs that had been slowly frozen to −25°C showed no after-thawing survival (Harvey, 1983). Both reports indicated that even at relatively higher subzero temperatures in general, very few fish embryos survive the freezing-thawing process. It is not indicated in these reports at what specific stages the developing embryos were subjected to freezing-thawing treatment, but it is apparent that the developmental stages make the search for a better after-thawing survival rate more complicated.

In 1987, the embryos of common carp at tail formation stage were frozen to −196°C and resulted in a 25% after-thawing survival and 18% hatching (Stoss and Donaldson, 1983). These results, to the best of our knowledge, are for the lowest temperature reached, and the latest embryonic stage employed, and constitute the highest survival and hatching rate obtained in the field of fish embryo freezing and preservation. The tail formation stage is comparable to the heart formation stage that we employed. We felt that the heart formation stage or post-heart formation stage may have prime significance in cryopreservation of embryos.

Post-thawing embryos with a beating heart mean a primordial circulatory system is functioning. The needed oxygen is easily provided, nutrients and waste can be exchanged. The circulatory system supported a continuing fast growing embryonic life and repair and replacement of damaged cells. Those survivals without hatching might mean that the damage caused by freezing-thawing exceeded a certain critical point and the effect showed up in following development. This phenomenon was proved to exist as latent injury (Sherman, 1967).

The coming task of our laboratory, using the advantage of spawning induction all the year round and based on the primary results, is to modify freeze-thawing procedure and rates of freezing and thawing to obtain better survival and hatching rate and lower freezing temperature.

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