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[Original Paper]

Cold Tolerance and Ice Nucleation Temperature of Medaka (*Oryzias latipes*) Embryos with Different Cryoprotectant Treatments

Padmanav ROUTRAY[1], Toru SUZUKI[1], Norihito KIMIZUKA[1], Kiyoshi KAWAI[1], Carlos Augusto STRÜSSMANN[2] and Rikuo TAKAI[1]

[1]*Department of Food Science and Technology, Tokyo University of Fisheries. Konan 4-5-7, Minato-Ku, Tokyo 108-8477*

[2]*Department of Aquatic Biosciences, Tokyo University of Fisheries. Konan 4-5-7, Minato-Ku, Tokyo 108-8477*

The cold tolerance and ice nucleation temperature of medaka embryos in three embryonic stages were investigated with different cryoprotectant treatments. The ice nucleation temperatures of the embryos in every stage showed a decreasing tendency by cryoprotectant treatment, in the order of trehalose, DMSO, and a combination of trehalose and DMSO. Further, the ice nucleation temperatures of the control embryos were depressed with the embryonic development. However, when the embryos were treated with the cryoprotectants, the stage dependency on the ice nucleation temperatures could not be recognized for 8 cell and optic bud stages, and only at the eyed stage the ice nucleation temperatures were significantly lower than those in early embryonic stages. On the other hand, the cold tolerance of the embryo at a temperature below 4℃, which was examined at the temperature range without occurrence of ice nucleation, became higher in every stage by cryoprotectant treatments. As for the stage dependency, it could be found that the cold tolerance of the embryos treated with cryoprotectants did not show a large difference among the stages, however, in the case of control embryo the cold tolerance at the exposure temperature below 4℃ showed an increasing tendency as the stage developed. As the conclusion, it was suggested that the lowering of the ice nucleation temperature of the embryos led to the increase in the survival rate of embryos at the cold temperature without ice nucleation, although other factors such as the development stage should be considered in.

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INTRODUCTION

Cryopreservation of fish embryos has not been successful until now. The main reasons are believed to be their large size, a multi compartment system (i.e., the yolk and blastoderm), low membrane permeability and a high sensitivity to low temperature or cold injury[1, 2].

[Key words : Cold tolerance, Medaka, Embryo, Nucleation temperature, Cryoprotectant；低温耐性，メダカ，胚，核生成温度，凍結保護物質]

Recent studies on *Drosophila melanogaster* embryos have suggested the importance of cold tolerance for the embryos in designing the cooling protocol[3]. Similar to *Drosophila embryos*, embryos of fish also show cold injury[4]. However, the exact mechanism of damage to fish embryos during cooling or freezing has not yet been identified due to lack of systematic research. Intra- and extra- cellular ice formation during freezing is not desirable to reduce the extent of extreme hypothermic, mechanical and osmotic stress on fish embryos[5, 6, 7]. Arii *et al*[8]. have reported that the medaka embryos survived at temperatures

as low as −40℃ for a short duration, but they did not make clear the low temperature injury and nucleation temperatures of embryos, which is important information to confirm the ice nucleation state in fish embryos. Liu *et al*[9]. showed that the ice formation within the egg, *i.e.* the intra-embryonic freezing, was the main factor affecting the survival of zebrafish (*Brachydanio rerio*) embryos. Recently, Zhang and Rawson[2] also reported the differences in cold tolerance of zebrafish embryos treated with different cryoprotectants. However, there are few reports on the nucleation temperature or cold tolerance of other fish species. Furthermore, though DMSO[10] and trehalose[11] have been used extensively as cryoprotectants, their effect on the nucleation behaviour of embryos has received little attention. The present study examined the nucleation temperature and cold tolerance of three embryonic stages of medaka (*Oryzias latipes*) embryos that were subjected to different cryoprotectant treatments.

MATERIALS AND METHODS

Embryo collection and incubation

For embryo collection, male and female medaka were reared in an aquaria at 25℃ with a 14 hour-light and 10 hour-dark-photoperiod and fed an aquarium fish diet (Kyorin) daily to satiation. The spawning took place daily under these conditions, usually within 1 hour of the onset in the light period. The eggs remained attached to the genital pore of the mother through the chorionic filaments, since no attachment devices or substratum was provided in the aquarium. The fish was taken out and immobilized with a net, and embryos were collected gently with a pair of forceps or a wide bored pipette. After collection, normal developing embryos were segregated under the microscope and transferred into groups of 25-30 to the petridishes for incubation in a 10 liter tank at 25℃ until the 8-cell, optic bud or eyed stages.

Treatment of cryoprotectants to embryos

Each time 25-30 live embryos of 2 -h (8 cell), optic bud or eyed stage were subjected to cryoprotectants, trehalose (0.5M) or DMSO (1.9M) for 20 minutes or a combination of trehalose (1M) for one minute and subsequently by DMSO (1.9M) for 20 minutes. The latter approach was based on the recent findings of Routray *et al.* (unpublished observations) that a short exposure to trehalose promoted the uptake of DMSO by fish embryos. The cryoprotectant treatments were carried out at room temperature of 20℃. The untreated group of embryos were used as control.

Determination of nucleation temperatures of embryos

The nucleation temperatures of untreated (control) and cryoprotectant-treated embryos of the three stages were determined using DSC (Differential Scanning Calorimetry) analysis. Prior to analysis, temperature calibration was carried out using pure water. Embryos (1.1-2.0 mg) were carefully blotted dry, weighed in aluminum DSC pans without sealing and then loaded into the Shimadzu (DSC-50, Kyoto, Japan) instrument at room temperature. The sample was then cooled at −2 or −5℃ per minute until −40℃. The obtained DSC curves were then analyzed using Shimadzu software (TA 60).

Cold tolerance of embryos

The cold tolerance test was carried out at five temperature regimes of 20℃, 4℃, 0℃, −5℃ and −10℃. Tolerance to low temperature was estimated in terms of the survival of embryos exposed to these temperatures. Groups of 25-30 embryos were transferred from 25℃ to 20℃ and subsequently to 4℃ to avoid temperature shock. Each embryo group was once kept at 4℃ for 30 minutes and then shifted to 0℃, −5℃ and −10℃. Both treated and control embryos were kept at the above temperatures for 24 hours. After these treatments the embryos were kept at room temperature 20℃ for 30 minutes and then washed repeatedly for 5 or 6 times in chlorine free water to remove the cryoprotectants from the embryos. The treated and untreated embryos were incubated for 10-11 days in water at a temperature of 25℃. The survival of embryos was determined by the

rate of hatching.

Statistical Analyses

The differences in the ice nucleation temperature among the embryos with various treatments were analyzed using analysis of variance (ANOVA)[12]. The survival rate of embryos under different conditions was compared with the Yates' continuity corrected Chi-square test. A probability value of $P<0.05$ was taken as statistically significant.

RESULTS

Nucleation temperatures of embryos

Figure 1 shows the typical DSC cooling curves of the embryos with different treatments for the three developmental stages. The difference in cooling rates of −2℃ and −5℃ per minute had no significant effect on the nucleation temperatures (Results not shown). Each DSC curve indicates a clear exothermic peak representing the ice nucleation. So the ice nucleation temperatures of the treated and control embryos in every stage, which were determined from the onset temperature of the peaks on the DSC traces, were listed in Table 1. It was noticed that the ice nucleation temperatures of the embryos were affected by both, the stage and cryoprotectant treatment. As the stage developed, the nucleation temperature of control embryos was depressed from −15.5℃ to −21.6℃. On the other hand, in the case of treated embryos, the nucleation temperature did not show large differences between 8-cell and optic bud stage embryos, however, it was significantly lower in the eyed stage. Furthermore, it should be noticed that by any treatment with DMSO, trehalose, or their combination, the ice nucleation temperatures of embryos in all stages were depressed significantly than those of the control.

Cold tolerance of embryos

As seen in Fig. 2, the survival rate of the control embryos without cryoprotectant treatment showed a significant decrease in all stages as the exposure temperature decreased. When the exposure temperature attained to −10℃ then the survival rates became 0% for all the stages. And also the survival rate of the control embryos showed a weak stage dependency that the survival increased with developing the stage, even though the difference between optic bud and eyed stages at the temperature below 0℃ was ambiguous. On the other hand, the embryos treated with DMSO, trehalose or their combination showed significantly higher survival rates at the temperature of below 4℃ in all stages than the

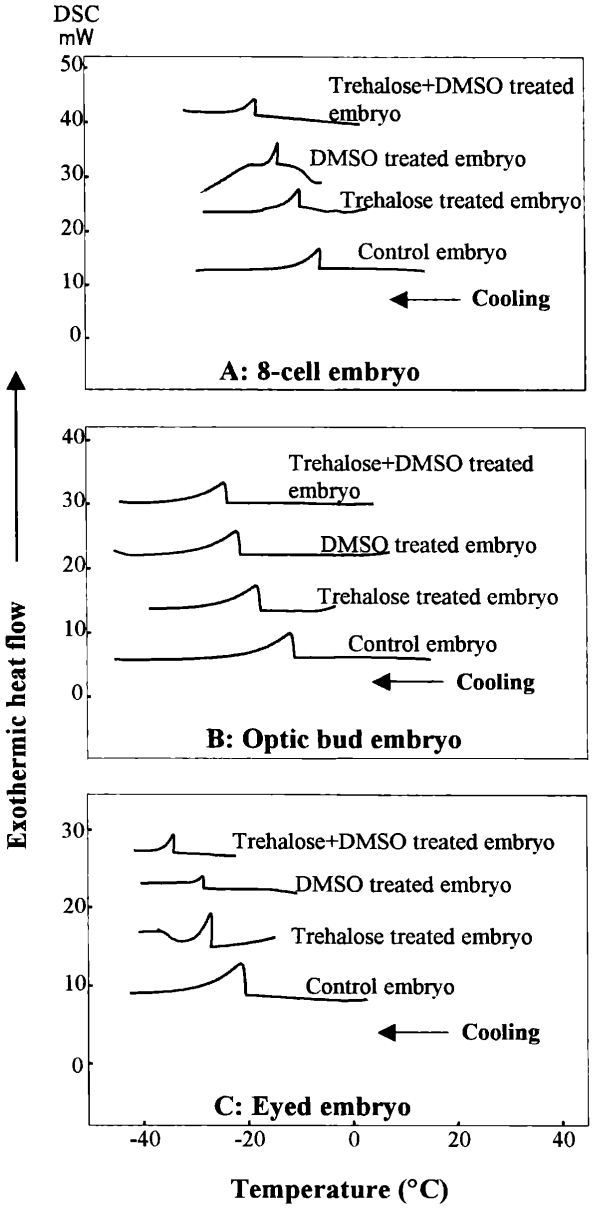

Fig. 1. DSC cooling curves showing freeze onset temperatures of the medaka embryos in three stages, A: 8-cell, B: Optic bud, C: eyed stage, with different treatments. The every scan rate was −2℃ per minute.

Table 1. Ice nucleation temperatures of medaka embryos subjected to different treatments. Data are shown as mean±SE (n=4). Values having different superscripts in a column or row differ significantly ($P<0.05$).

Embryo stage	Control	Trehalose	DMSO	Trehalose + DMSO
8-cell	-16.5 ± 0.5^{a}	-18 ± 0.9^{c}	-21 ± 0.4^{d}	-25 ± 1.2^{e}
Optic bud	-17.1 ± 0.8^{a}	-18.1 ± 0.9^{c}	-21.6 ± 1.0^{d}	-25 ± 0.3^{e}
Eye	-21.6 ± 0.5^{c}	-25.4 ± 0.5^{d}	-27.6 ± 0.8^{e}	-31.1 ± 1.5^{f}

control. However, only at the exposure temperature of 20℃, reversibly, the survival rates of the treated embryos were less than those of the control embryos. Further, in case of the treated embryos, the effect of the embryonic stage on the survival rate seemed to be negligible entirely at the exposure temperature below 4℃, whereas the survival rates of the control embryos decreased slightly with developing the stage. When examined in detail, it was found that the eyed stage embryo treated with DMSO or trehalose+DMSO showed a lower value of the survival rate only at 20℃ than other stage embryos. Further, it should be stated that the embryos treated with DMSO had a better survival rate than those with trehalose.

DISCUSSION

Liu *et. al.*[9] reported that the ice nucleation temperature of the zebrafish embryo shifts to lower temperatures as the stage develops. Our experimental results with medaka embryos also provided similar results. This result may be explained as follows: the different organs in the embryo develop with progression of the stage, so that the water in the embryo interacts with an increased interface such as the membrane and/or is trapped in small size compartments. It is known that the homogeneous ice nucleation temperature of pure water in small size emulsion is depressed to −40℃ when the size is 10 micro meter in diameter[13]. Although the nucleation in embryos may be not homogenous nucleation, the ice nucleation temperature depressing of the developed embryo, especially of the eyed stage, might be also due to decrease of the compartment size. On the other hand, the ice nucleation temperatures of the embryo in all embryonic stages were lowered significantly after treatments with DMSO or trehalose, alone or in a

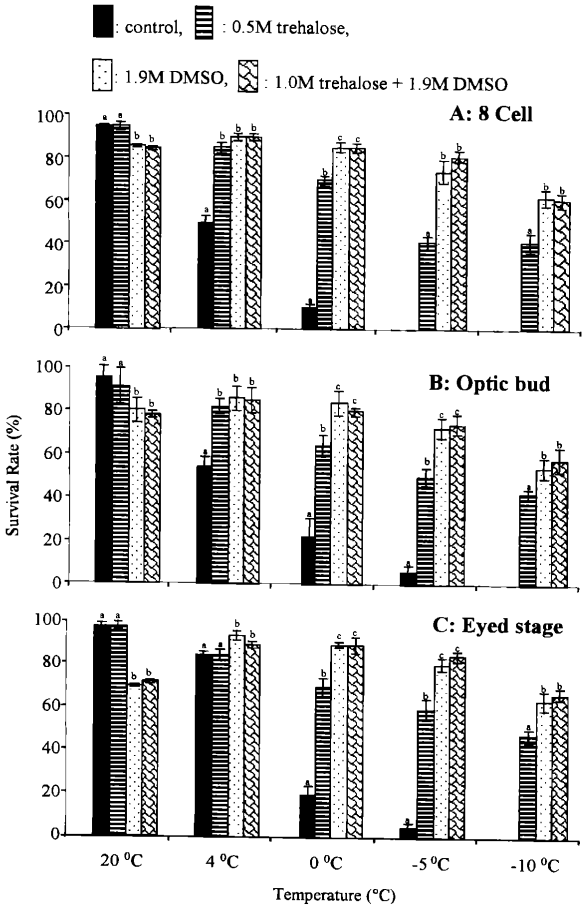

Fig. 2. Survival rates (%) of medaka embryos in different temperature regimes, A: 8-cell, B: optic bud, C: eyed stage with different treatments. Data are shown as mean (SE (n=4). Bars having different superscripts differ significantly in a temperature group ($P<0.05$).

combination, in comparison to untreated controls. DMSO is a membrane permeable cryoprotectant while trehalose is not. As to the role of trehalose, the following hypothesis could be considered: In the embryos treated with trehalose, the freezable, free water is thought to have decreased by dehydration. This would correspond to decreasing the volume of subjected water. Just as an emulsified water droplet, the smaller the volume of water is, the lower the possibility of ice nucleation becomes.

As a result, the ice nucleation in the embryos treated with trehalose does not easily occur. This study did not compare with other saccharides or membrane impermeable cryoprotectants, however, the same effect would be expected if above hypothesis is true. On the other hand, by the treatment with DMSO or DMSO plus trehalose, the concentrations of DMSO in the embryo are considered to have increased up to 0.3M to 0.6M according to our previous study[14]. Such an increase of the DMSO concentration might lead to not only an equilibrium melting point depression but also the ice nucleation temperature depression since the similar phenomena have been confirmed on many aqueous solution systems emulsified in inert oil[15]. However, the reason is not understood, presently. At least it was confirmed that the ice nucleation in the embryos has not occurred in the range of the exposure temperature to −10℃ on our cold tolerance test. Therefore, it can be said that the cold tolerance as discussed below was not affected by the intracellular ice nucleation of embryo, but the role of other factors should also be taken into consideration.

In the absence of cryoprotectant, almost all the medaka embryos without the effects of the embryonic stage received a significant damage at low temperatures below 4℃ to −10℃ where no ice nucleation occurred, though the eyed stage embryo showed stronger tolerance at 4℃ than the other stages. The fact which a fish embryo is damaged at low temperature without ice nucleation has been also confirmed for the embryos of zebrafish[1,2] and common carp, *Cyprinus carpio*[16] in early developmental stages. As described in the results, in the presence of cryoprotectants the cold tolerance of embryos in every stage indicated a remarkable increase. While, cryoprotectants also affected the depression of the ice nucleation temperatures of the embryos. Thus, to explore the relationship, as an example, the nucleation temperature versus the survival rate at 0℃ for these three stages of the embryo was plotted in Fig. 3. From the figure it was found that the survival rate of any stage at least 0℃ showed a trend of increase with depression of the ice nucleation temperature though it attained to be constant, i.e. 70% when the nucleation temperature was very low. It is interesting that the trend curve coincided closely between optic bud and 8cell stages while the trend curve of the eyed stage embryo shifted to lower temperature without changing of the curve formula. This may mean that the cold tolerance of the embryo under no ice nucleation condition could be determined, not only by deep undercooling ability represented as ice nucleation temperature, but also by other factors induced from developing of the stage. The other factors, however, are unknown at the present stage. The ice nucleation temperature of aqueous solution has been reported to be proportional to the equilibrium melting point which is depressed with an increasing on the concentration of solutes[15]. When such a phenomenon is considered relating to the stabilizing effect of water molecules due to the addition of a solute, the torelance of an embryo to low temperatures may be gained through water stabilizing. Additionally, in the present study, although the nucleation temperature was successfully lowered to −30℃, the cold tolerance tests, for longer hours, could not be carried out beyond −10℃ because the ice nucleation from the solution-surrounding the embryo occurred frequently.

Cold injury without ice nucleation, which is similar to the high risk of intracellular ice formation, should be recognized to be the major obstacle in the way of successful cryopreservation of medaka embryos. This study might help, not only in finding new avenues for successful cryopreservation of fish embryos, but also in

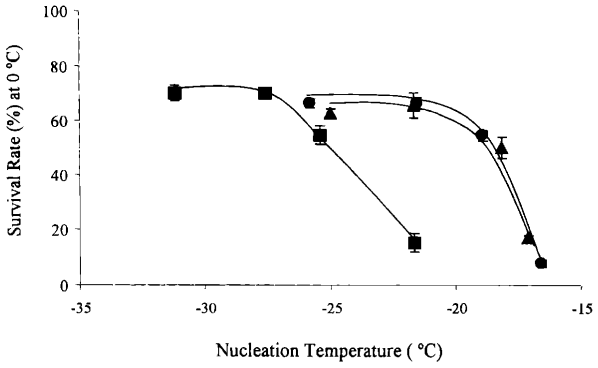

Fig. 3. General trend showing the relation between nucleation temperature and survival of embryos at 0℃. ■: 8-cell, ▲: optic bud and ●: eyed stage. Data are shown as mean±SE.

non-freezing storage of fish embryos for a short time.

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