Possibility of cryopreservation of medaka eggs using liquid meniscus

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Abstract
The cryopreservation of fish eggs is an important subject in the field of fishery and preservation of biological species. Thus far, there has been no success in the preservation of fish eggs because of the large size of the eggs and the thick external shell. This paper discusses the effectiveness of using the liquid meniscus formed around the egg for protecting its morphology. Freezing and thawing experiments of medaka eggs were performed under different freezing conditions, and the hatching rate of the egg was examined. Before freezing, the eggs were dehydrated at room temperature in order to reduce the effect of volume expansion caused by freezing. It was confirmed that 100% of the eggs dehydrated by 15% or less were successfully hatched. In the freezing process, a medaka egg was placed on a hydrophobic cooling plate and a thin liquid meniscus was formed around the hydrophilic egg surface. An aqueous solution of trehalose was used as the liquid meniscus as well as a cryoprotectant to prevent damage caused by freezing. Cryopreservation of the egg was not successfully performed for all processes, including intracellular freezing; however, 80% of the eggs were alive even after freezing of the external meniscus. Therefore, it is confirmed that the liquid meniscus is effective for the cryopreservation of the external shell. The liquid meniscus can reduce the physical stress due to extracellular ice growth. Moreover, since the liquid meniscus system has a low heat capacity, the thermal process is easy to control compared to the conventional method. We concluded that the present method can be used for the cryopreservation of fish eggs.

Key words : Fish egg, Cryopreservation, Liquid meniscus, Dehydration, Medaka

1. Introduction

The cryopreservation of a living material is a promising method for bridging temporal and spatial gaps in many applications such as medical treatment, food processing, and stockbreeding. Many studies have been conducted on cryopreservation, and a preservation protocol has been developed for small tissue cells, including the embryos of mammalian or oocytes of animals. The sperm of fish can be relatively easy to cryopreserve. However, the survival of fish eggs after cryopreservation has not yet been achieved (Edashige et al., 2006). Compared to the eggs of higher vertebrates, fish eggs are large and contain a large amount of yolk protected by a hard outer shell called the chorion. According to the morphological data of the medaka egg presented by Shirakashi et al. (2012), the chorion is 15-μm thick and has a characteristic tree-ring-like structure. In addition, the yolk envelope has a double-membrane structure. This complicated structure makes it difficult to introduce a cryoprotectant into the fish egg owing to the low permeability. Therefore, it is difficult to apply the conventional technique using ultra-rapid cooling in combination with a high internal concentration of cryoprotectants for vitrification.

Despite these technical difficulties, the cryopreservation of fish eggs is a desirable method for conserving commercially important and endangered fish species, thus ensuring a stable supply for the aquaculture industry and allowing for storage of generic materials for future use. Consequently, many trial studies have been conducted for

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impregnated embryos with cryoprotectant after dechorionation, i.e., for blastomeres of whiting, pejerry, medaka (Strussmann, 1999), medaka oocytes (Valdez, 2013), zebrafish ovarian follicles (Godoy, 2013) as well as for the cryopreservation of spermatogonia (Lee, 2016). Moreover, in order to achieve effective permeation of the cryoprotectant into the fish egg and embryo, an electroporation technique was developed (Shirakashi et al., 2004, 2015; Rahman et al., 2013).

With regard to cell damage during the cryopreservation of mouse oocytes, Mazur et al. (2005) summarized the effects of extracellular ice on the intracellular ice formation. In a general cooling procedure, the cryoprotectant solution containing a suspension of oocytes or eggs is first frozen; this can cause mechanical damage owing to the extracellular ice growth. In the case of slow cooling for achieving extracellular freezing, denaturation may occur because of a long operation period. These issues have not been resolved yet, and a suitable cryopreservation technique has not yet been developed.

One of the authors proposed a new freezing method that combines the use of a liquid meniscus covering the hydrophilic egg surface and predehydration processing (Tsuruta, 2012). Covering the outer surface of the egg with a thin liquid film of cryoprotectant in the form of a liquid meniscus is effective for preserving the egg morphology during freezing because the mild solidification of the meniscus can be performed without mechanical stress from the outside. The use of a liquid meniscus has another advantage in that the freezing operation is easy to control because the liquid meniscus system has a much lower heat capacity than the suspension form. The freezing method has been applied to salmon eggs 10 times larger than the medaka egg, and we confirmed that the existence of a shape preservative effect after thawing (Kido and Tsuruta, 2013). Based on the findings that the protection of chorion has an important role in the preservation method, we started the experiments using medaka eggs to show the effectiveness of a liquid meniscus in the cryopreservation of fish eggs. Before our trial, Ujihira et al (1995) conducted a similar experimental study on the cryopreservation of medaka eggs with use of a liquid meniscus formed by a suspended droplet of DMSO-water mixture. They clarified the effect of thawing rate on the cryopreservation of egg morphology. In our studies, we applied a smaller droplet on the hydrophobic surface. The cooling procedure consisted of four processes: predehydration, precooling, meniscus freezing, and intracellular freezing. Currently, we have not been able to cryopreserve medaka eggs by following all the processes. In this study, therefore, detailed experiments are carried out to confirm the process causing cell damage and discuss a modification method.

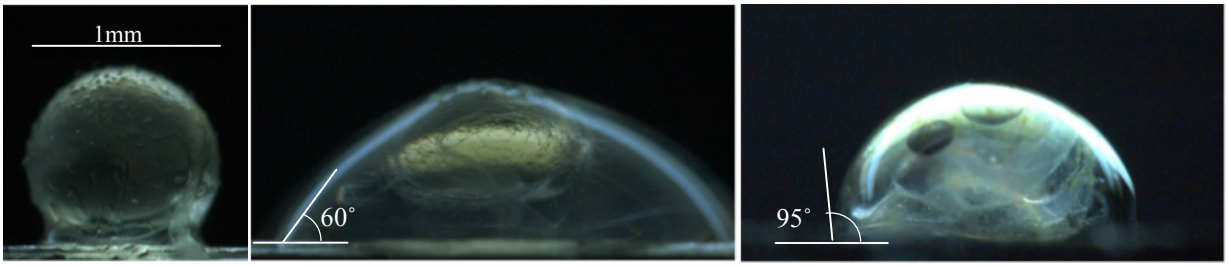
2. Experimental apparatus and method

2.1 Collection of medaka eggs

Male and female medaka, purchased from a local fish dealer, were reared in aquaria at 27 °C under 14-h light and 10-h dark periods. Naturally fertilized eggs were collected after spawning. Usually, the spawning took place daily within one hour from the onset of lighting. Since the eggs remained attached to the genital pore of the female medaka, we removed the medaka using a net and collected the egg gently with a pair of forceps. Embryos at Iwamatsu stages (Iwamatsu, 2004) from three (two blastomeres) to four (four blastomeres) were the subjects of the experiments in this study.

2.2 Formation of liquid meniscus around medaka eggs

Owing to surface tension, the shape of the water surface changes in the vicinity of the vertical wall, and its maximum height at the hydrophilic wall can be given by the Laplace length $l_p = \sqrt{2\sigma/\rho_l g}$. Since the Laplace length



(a) medaka egg (b) SUS surface (c) CYTOP surface

Fig. 1 Medaka egg and liquid meniscus of 25% trehalose solution formed on substrate with different wettability.

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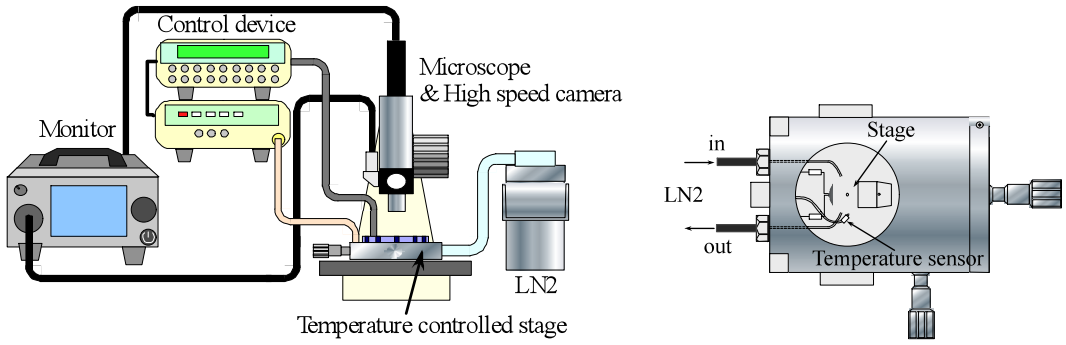
is estimated to be approximately 4 mm for 0 °C water and the egg surface is hydrophilic, the 1-mm diameter medaka egg can be completely covered by water. The difference in wettability between the egg surface and the substrate forms a thin liquid meniscus around the medaka egg. As shown in Fig. 1, the contact angle when SUS is used as the holding substrate is approximately 60°; 4 μL of solution is required to cover the entire egg. As a result, a thicker liquid layer is formed at the lower portion of the fish egg, which increases the possibility of formation of a large ice crystal. On the other hand, when CYTOP (Asahi Glass Co. Ltd.) is coated to the Si substrate, the droplet volume is reduced to 2 μL. The contact angle is 95°, and it is observed that the liquid can form a thin film over the entire egg and cover it uniformly. As stated before, Ujihira et al. (1995) used a droplet suspended from a metal probe to cover an egg. They reported that the volume of the hanging droplet was 9 μL.

As a cryoprotective solution, we use trehalose aqueous solution in this study. From the prior experiment, a concentration of 25% was selected to suppress the growth of ice crystals and to reduce the mechanical stress on the egg. The study showed that the trehalose solution increased the hatching rate of the medaka egg (Sano et al., 2014). The details are going to be presented elsewhere.

2.3 Experimental apparatus

To observe the freezing and thawing behaviors of medaka eggs covered with a liquid meniscus, we used a programmable cooling and thawing stage system (Linkam 10008) combined with a digital high-speed microscope system (Keyence VW-6000). The system is shown in Fig. 2. To freeze the sample at the observation stage of 21 mm diameter, liquid N_2 was supplied as shown in Fig. 2(b). An electrical heater and thermal sensor are arranged in the stage to control the cooling rate using the digital program regulator. The maximum cooling rate was 100 °C/min



(a) Cooling stage and observation system (b) Linkam stage

Fig. 2 Experimental apparatus for cooling and thawing.

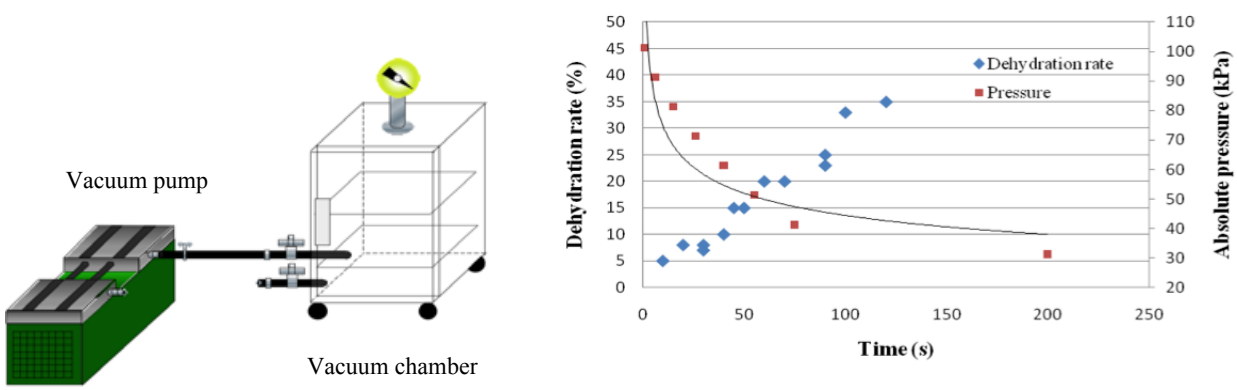


Fig. 3 Vacuum dehydration system and relation between dehydration rate and vacuuming time.

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in the catalog data, but 85 °C/min in practice when a medaka egg is used. We used this system for thawing at a heating rate of 5 °C/min.

Before cooling, for the predehydration of medaka eggs, we used a small vacuum chamber equipped with a vacuum pump, as shown in Fig. 3. It was confirmed that decreasing the water content inside the seafood could lower the freezing point temperature and the size of the ice crystal formed inside the cell tissues (Hamidi and Tsuruta, 2008). Dehydration before cooling can help reduce the damage caused by intracellular ice formation. In the present experiment, an egg just collected from the medaka was placed in the vacuum chamber at room temperature, after which evacuation started. After the initiation of vacuuming, the moisture content of the medaka egg started to decrease. Figure 3 shows the dehydration rate and the chamber pressure as a function of the vacuuming time. The dehydration rate was defined by the ratio of the dehydrated mass of water to the initial mass of the egg. The amount of dehydrated water was obtained by measuring the weight loss of the egg after vacuuming was stopped at the time of data point in Fig. 3. Based on this relation, we fixed the dehydration rate of the sample eggs and examined the effect of the dehydration rate on the hatching rate of medaka eggs.

2.4 Experimental method for cooling and thawing

At first, vacuum dehydration was carried out for the egg before the freezing operation, as explained previously. The dehydration rate was changed up to 35% in weight.

The processes of cooling and thawing are shown schematically in Fig. 4. One medaka egg was placed on the CYTOP-coated Si plate and covered by the liquid meniscus, which was set on the microscope stage having a function of temperature control with cooling and heating. After moderate cooling at room temperature to 10 °C (a–b), the egg was cooled slower (at a rate of 2 °C/min) down to -3 °C (b–c), and ice seeding was performed on the liquid meniscus (c–d) with a fine needle cooled by the liquid nitrogen at -196 °C. It is important to induce phase change in the meniscus only, because the chorion should be protected by the solid phase of the meniscus before internal freezing. For lower seeding temperatures, meniscus freezing was accompanied by internal freezing. Therefore, the present ice seeding conditions, the slow cooling rate of 2 °C/min, and the seeding temperature of -3 °C, were determined after several trials as shown later. Following meniscus freezing, the egg was immersed into the liquid nitrogen and quenched at approximately 3400 °C/min to vitrify or promote small ice-crystal formation (d–e). Thawing was carried out on the observation stage at 5 °C/min again (e–f). Finally, we examined the hatching rate of egg in an incubator.

In addition to ultra-rapid cooling with the use of liquid nitrogen, we examined the effect of internal freezing on the hatching rate by changing the cooling condition. Figure 5 shows four patterns of cooling and thawing operations carried out in this study. The thawing rate was the same at 5 °C/min, but the cooling rate and temperature were different in the four cases. The experiments were carried out for eight samples under the same conditions, and the hatching rate was obtained. In addition, to understand the internal freezing temperature of medaka eggs under slow cooling conditions, a T-type thermocouple of 0.1-mm diameter was installed in such a way that it was in contact with the egg surface.

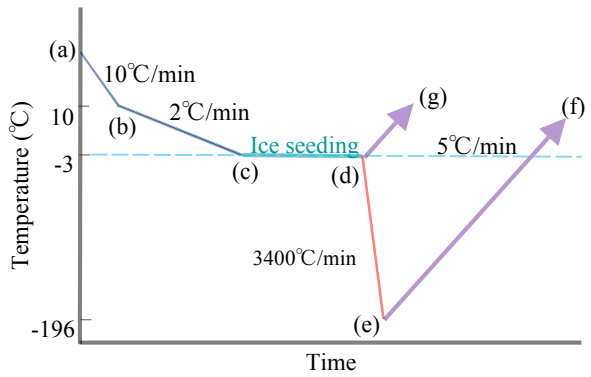

Fig. 4 Cooling and thawing processes.

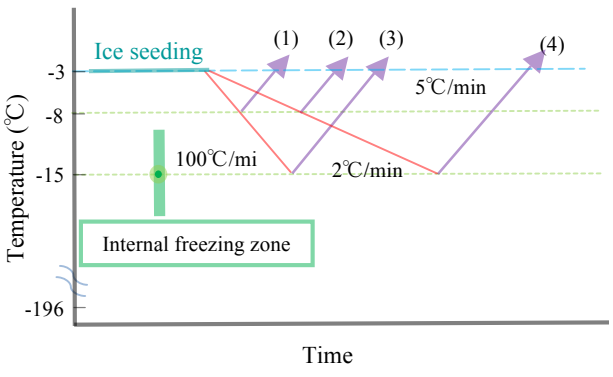

Fig. 5 Trial tests on cooling effects.

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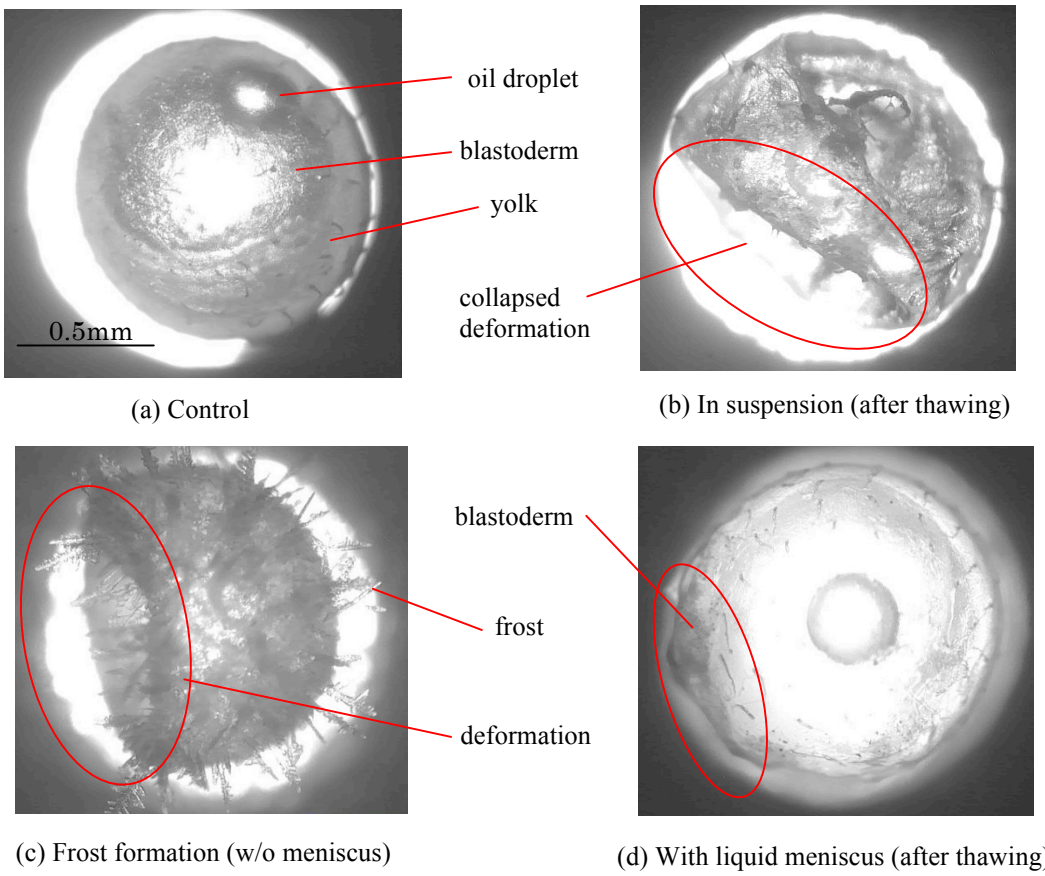

Fig.6 Appearances of medaka eggs in different processes.

3. Results and discussions

3.1 Effect of the liquid meniscus on preservation of egg morphology

First, we present the effectiveness of the liquid meniscus for the preservation of the morphology of medaka eggs. Figure 6 shows the comparison of the appearances of medaka eggs under different conditions. Figure 6 (a) is the control that is a fresh egg containing an oil droplet, blastoderm, and yolk. As for a reference, we performed a freezing/thawing experiment of the egg in the form of a suspension with the use of the trehalose aqueous solution. The cooling and thawing rates were set at 85 °C/min and 5 °C/min by the Linkam stage, respectively. The thawing process was started at -40 °C. As shown in Fig. 6 (b), the collapse was observed after thawing, which is induced by extracellular ice formation. In the case of cooling without the liquid meniscus or the outer liquid suspension, frost formation occurred at the egg surface, as observed in (c). The micrograph was taken during the cooling process before the occurrence of internal freezing. Deformation was observed at the left part, but the reason for this is unclear at present. When we used the liquid meniscus, frost formation was suppressed owing to the egg surface being covered with a mushy-like ice phase. This allowed us to preserve the egg morphology after freezing/thawing. Figure 6 (d) shows the result after thawing through all the processes shown in Fig. 4 for a 15% predehydrated egg. We observe that the meniscus can preserve the configuration of the medaka egg. The blastderm was also observed but the egg did not hatch.

3.2 Examination of the occurrence of fatal damage during the process

In the present study, the cryopreservation of medaka eggs could not be successfully performed by following all the processes (a–b–c–d–e–f) proposed in Fig. 4. Therefore, we are attempting to investigate which process caused fatal damage.

First, we examined the effects of predehydration on the hatching rate. Figure 7 (a) shows the hatching rates of eggs at different dehydration rates. The number of samples studied was 15 for each dehydration rate. It is clear that

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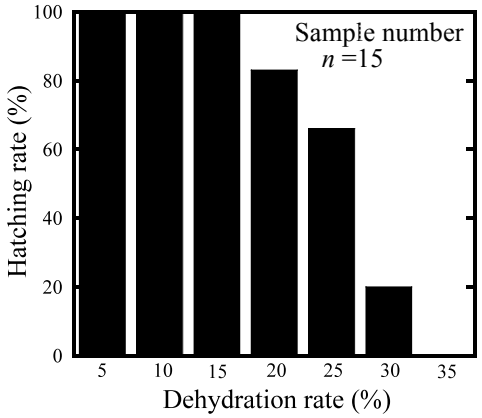
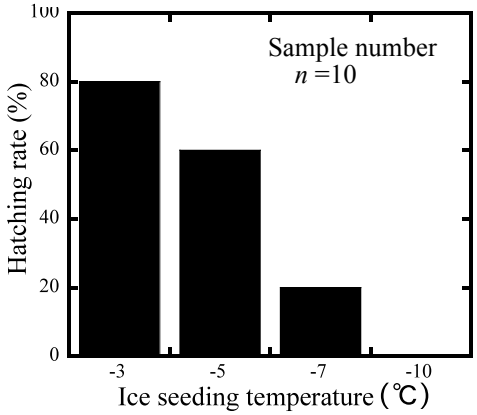
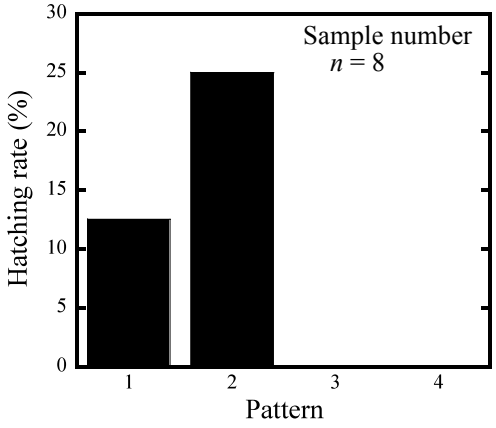


(a) Effect of dehydration rate



(b) Effect of freezing temperature



(c) Effect of cooling processes

Fig. 7 Hatching rate after several stage of process.

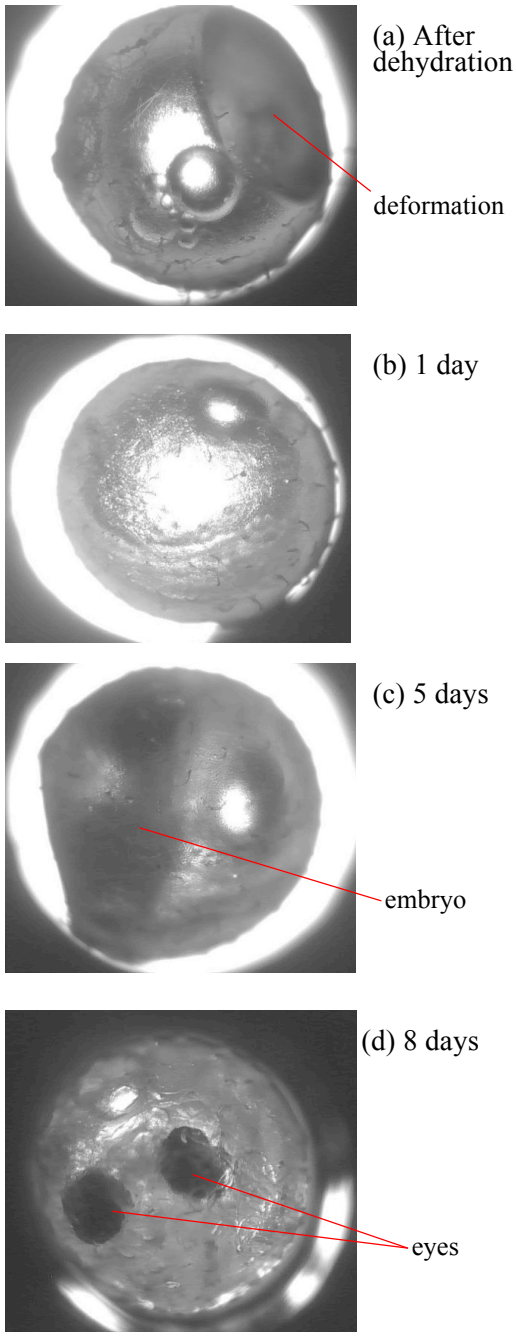


Fig. 8 Embryotic development process of dehydrated egg.

predehydration does not affect hatching for dehydration rates up to 15%, although a larger dehydration reduces the hatching rate. We observed the process of embryonic development for the case of 15% dehydration, as shown in Fig. 8. A small deformation due to the dehydration was observed at the right corner in (a), but the egg recovered its original shape in one day (b). The embryo differentiated further and two eyes were clearly observed in (d). After 13 days of dehydration, the egg hatched. The results indicate that a predehydration rate of 15% did not cause any damage in the present study. We selected 15% as the dehydration rate in the following tests.

Second, the effect of ice seeding temperature on the hatching rate was studied by conducting the operation (a–b–c–d–g) in Fig. 4, excluding the internal ice formation. Ice seeding with the use of a cold needle initiated

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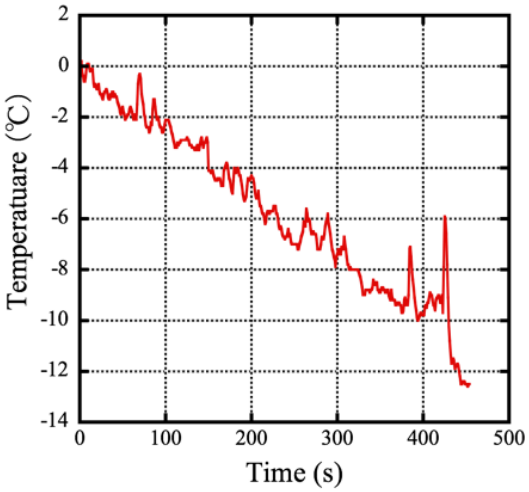

Fig. 9 Temperature transient showing the intracellular freezing.

meniscus freezing. In the case of ice seeding at -3 °C, Fig. 7 (b) shows that 80% of the eggs were hatched after thawing the frozen meniscus. This interesting result clearly indicates that the eggs are still alive even after freezing the meniscus. By lowering the freezing temperature, the hatching rate decreases, and meniscus freezing at -10 °C resulted in no survival. Since chilling injury or rapid solidification may be considered a reason for damage, meniscus freezing at -3 °C is concluded as the best solution for protecting the chorion.

From the above examinations, it is considered that internal freezing can cause fatal damage to eggs in the present study. Finally, we examined the effect of the cooling process after ice seeding. As explained in Fig. 5, we carried out four types of cooling tests after meniscus freezing. The cooling rates were set at 2 °C/min and 100 °C/min, and the cooling-down temperatures were changed to -8 °C and -15 °C. Figure 7 (c) shows the results of the hatching rate in the processes of four patterns of cooling operations. In cooling patterns (3) and (4), where the eggs were cooled down to -15 °C, we obtain a zero hatching rate. On the other hand, when we stop the cooling at -8 °C, a small number of eggs were still alive in process patterns (1) and (2). Since the cooling temperatures of both cases were -8 °C, it is necessary to confirm whether internal egg freezing occurs at -8 °C.

The temperature transients during the cooling process of the dehydrated eggs were measured using the T-type fine thermocouple; these transients are shown in Fig. 9. The cooling rate was 2 °C/min, the same as the case of pattern (2) in Fig. 5. A marked temperature rise is observed at approximately -10 °C during the cooling. Since the visual observation also suggested the occurrence of internal freezing at -10 °C, this temperature rise is considered to be caused by the latent heat release owing to internal egg freezing. Therefore, it is suggested that the internal freezing of eggs requires a larger super cooling than -8 °C. This result indicates the possibility of nonfreezing inside the egg in the case of cooling pattern (1) and (2) in Fig. 5.

4. Conclusions

The cryopreservation of medaka eggs was not successfully performed for all processes, including intracellular freezing; however, the morphology of medaka eggs was preserved with the aid of a thin liquid meniscus formed around the eggs on the hydrophilic surface. Moreover, it was also found that 80% of the eggs were still alive even after freezing of the external meniscus when ice seeding was carried out at -3 °C for 25% concentration of trehalose solution covering the 15% predehydrated eggs. Therefore, it was verified that the liquid meniscus was effective for the cryopreservation of the egg chorion. This study clarified that fatal damage occurred because of intracellular ice formation. Further modifications and trials are necessary for the method using the liquid meniscus to prevent internal damage.

Acknowledgment

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