Reprinted from

THERIOGENOLOGY

Theriogenology 58 (2002) 1483–1496

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Padmanav Routray[a], Toru Suzuki[a,*], Carlos Augusto Strüssmann[b], Rikuo Takai[a]

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

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

Received 1 October 2001; accepted 9 January 2002

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

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

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Abstract

High performance liquid chromatography (HPLC) was used to assess the uptake dynamics of the cryoprotectant DMSO by intact unfertilized eggs (stage 0), 8-cell (stage 5) and eyed embryos (stage 30) of medaka, *Oryzias latipes,* the relation of the internal concentration (C_{in}) of DMSO with fertilization and survival rates, and the effects of several factors on these processes. The factors examined were: cryoprotectant concentration (0.6, 1.2, 1.9 and 2.5 M), impregnation time (1, 3, 5, 10, 15 and 20 min), temperature (0, 5 and 20 °C), hydrostatic pressure (0 and 50 atm), and the osmotic conditions of the materials (normal or partially dehydrated). Cryoprotectant permeation, estimated from the initial rates of DMSO uptake, was higher in embryos than in eggs and increased with embryonic development; however, the DMSO C_{in} in eyed embryos reached a plateau at 1–5 min and could not be increased by prolonging impregnation. The highest fertilization and survival rates for any given DMSO C_{in} were obtained with high concentrations and short times of impregnation rather than low concentrations and long impregnation times. Application of hydrostatic pressure (50 atm) and exposure for 3 min to a 1 M trehalose solution prior to impregnation induced a substantial increase in the DMSO C_{in} of 8-cell embryos in comparison to untreated controls with no significant effect on survival. Hydrostatic pressure also promoted DMSO uptake in unfertilized eggs, but with rapid loss of viability, and was ineffective in eyed embryos. The uptake of DMSO and its toxicity to 8-cell embryos were directly proportional to the temperature of impregnation. The results of this study reveal important interactions between cryoprotectant concentration, impregnation time and the developmental stage (or type) of the materials and provide evidence that hydrostatic pressure, temperature of impregnation and the osmotic conditions of the materials can be manipulated to increase the uptake of cryoprotectant by fish eggs and embryos.

Keywords: Fish; Egg; Embryo; Cryoprotectant impregnation; Medaka (*Oryzias latipes*)

* Corresponding author. Tel.: +81-3-5463-0623; fax: +81-3-5463-0585.
E-mail address: toru@tokyo-u-fish.ac.jp (T. Suzuki).

0093-691X/02/$ – see front matter
PII: S0093-691X(02)01076-2

1. Introduction

Several highly effective cryopreservation protocols have been developed for mammalian oocytes and embryos [1–3]. The most common protocols include impregnation of the cells with a permeating cryoprotectant such as DMSO prior to cryopreservation. The presence of this chemical, like other permeating cryoprotectants, reduces the gap between freezing and vitrification temperatures and thereby prevents fatal, widespread intra- and extracellular ice formation [4]. Cryopreservation protocols based on data for mammalian embryos have been tested in isolated cells such as blastomeres [5–7] and whole eggs and embryos [8–10] of fish, but successful cryopreservation was obtained only with the isolated cells. The difficulty with whole fish eggs and embryos apparently lies in the peculiar characteristics of these biological materials. For instance, fish egg and embryos are several times larger than those of mammals, have a thick chorion, a large volume of yolk segregated from the active cytoplasm, and show considerable structural complexity early in development [10–14]. Therefore, it is very difficult to achieve a swift and uniform permeation of the cryoprotectant into the various compartments of fish eggs and embryos, resulting in toxicity to some cells while in others the concentration of cryoprotectant is not sufficient to prevent cryoinjuries. Researchers have tried to circumvent these problems in several ways including the impregnation of eggs and embryos with cryoprotectant after dechorionation [15], under negative pressure [16], or by direct delivery of cryoprotectant into the yolk by microinjection [17] but as observed in earlier studies, failed to achieve successful cryopreservation. It must be noted also that isolated cell cryopreservation, although technically feasible, is not practical in large-scale hatchery activities; in these situations, it is essential to avoid the use of sophisticated equipment and time consuming techniques [18]. These facts underscore the need for fundamental information on the factors that affect the uptake of cryoprotectants in order to develop successful protocols for intact fish egg and embryo cryopreservation [8,10].

In a study of DMSO permeation into intact fish embryos, Suzuki et al. [19] measured the concentration of the cryoprotectant in the embryos and proposed a model to predict the internal concentration and the instantaneous survival rates as a function of the external concentration. However, their model did not take into account the stage of development of the embryos and the duration of exposure to the cryoprotectant, two factors that have been shown to influence the membrane permeability and the tolerance of the cells to the cryoprotectant [18–21]. Other factors can also promote or impair the uptake of cryoprotectant in biological systems. It is known, for instance, that hydrostatic pressure affects the transport mechanisms and membrane permeability in cells and tissues [22–24]. In addition, cells will conceivably absorb extracellular water and waterborne permeating substances more readily if they are partially dehydrated before impregnation, as has been experimentally demonstrated in fish sperm [25]. Finally, the temperature of impregnation has been shown to influence greatly the uptake of cryoprotectant in mammalian embryos [26,27]. Nonetheless, the potential relevance of these factors for fish egg and embryo cryopreservation has been largely neglected. The study of hydrostatic pressure, for instance, is limited to a report by Leung and Jamieson [16] on the effects of mild vacuum during impregnation.

The objective of the present study was to investigate the potential of several factors to affect the uptake of DMSO by fish eggs and embryos using the medaka, *Oryzias latipes*, as

the experimental model. Thus, the internal concentration of DMSO in the materials, measured by high performance liquid chromatography (HPLC), was examined in relation to the external concentration of the cryoprotectant, the duration of impregnation, the hydrostatic pressure, temperature, developmental stage (unfertilized eggs and different stages of embryos) and the osmotic conditions of the materials during impregnation. We also investigated the toxicity of DMSO to the eggs and embryos in relation to these factors in order to determine acceptable levels of viability after impregnation and before cryopreservation.

2. Materials and methods

2.1. Collection of eggs and embryos

The wild-type medaka brood stock used in this study was purchased from a local fish dealer. Fish were reared either in pairs (one female and one male) or in larger groups in 25 l aquaria kept at 25 °C and under a 14-h light and 10-h dark photoperiod, but with no spawning substratum. Fish were fed an aquarium fish diet (Medaka feed, Kyorin Food Co., Himejishi, Hyoogo-Ken, Japan) daily to satiation. Individual females spawned consecutively for many days, usually within 1 h of the onset of the light period. For collection of unfertilized eggs (stage 0) [28] actively spawning females were separated from the males a day prior to the experiment in order to prevent spontaneous spawning, and humanely killed about 30 min before the expected spawning time. Ovaries were excised and mature oocytes were immediately removed from the follicle layer using a pair of precision forceps. Fertilized eggs, which remained attached to the mother by their chorionic filaments, were collected using either a forceps or a wide bore pipette after gently restraining the mother with a hand net. After collection, normally developing embryos were selected under a microscope and transferred to petri dishes for incubation until the 8-cell (stage 5) [28] and eyed embryo (stage 30) [28] stages.

2.2. Hydrostatic pressure, osmotic pressure and temperature treatments

For pressure treatment, about 13–30 unfertilized eggs, 8-cell or eyed embryos were transferred to 2 ml Eppendorf tubes containing 0.6, 1.2, 1.9, and 2.5 M DMSO in Yamamoto Ringer (YR) solution [29]. The tubes were immediately closed and inserted into a water-filled 20 ml disposable injection syringe, the tip of which had been sealed by heating. Care was taken to leave no air bubbles trapped inside the tubes or in the syringe before insertion into a pressure vessel filled with high-pressure mobile oil (Fig. 1). This procedure was completed in approximately 1 min. A hydrostatic pressure of 5.15×10^3 kPa (50 atm) was then applied for 1, 3, 5, 10, 15 or 20 min at a temperature of 20 °C using a pressure pump (Riken Power UP228, Riken Seiki Co. Ltd., Tokyo, Japan). This choice of pressure was based on preliminary studies that showed the occurrence of malformations or low survival at higher hydrostatic pressures (Routray et al., unpublished results). Groups of 10 to 20 8-cell embryos were used in the trials to examine the effects of impregnation temperature and osmotic conditions of the materials on the uptake of DMSO.

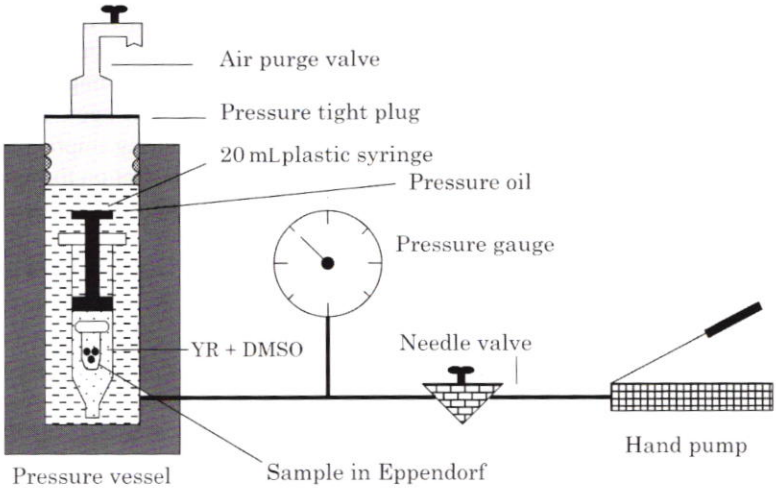

Fig. 1. Schematic diagram of the equipment used for hydrostatic pressure treatment.

During osmotic manipulation, embryos were first subjected to a 1 or 3 min partial dehydration in a 1 M solution of the disaccharide trehalose and then impregnated with 1.2 M DMSO in YR solution for the same periods as with pressure treatment. Trehalose was chosen because it permeates slowly and also helps to stabilize the membrane structure [30]. The degree of dehydration of the embryos was not quantified but the chorion was visibly shrunk after the initial treatment with trehalose. The shape of the chorion returned to normal upon immersion in the DMSO solution. Osmotic manipulation was performed at 20 °C. Three temperature regimes, 0, 5 and 20 °C (±0.1 °C) were used to test the effect of impregnation temperature on DMSO uptake. For this purpose, embryos were immersed in a 1.5 M solution of DMSO and kept in a precision incubator (Sanyo SRR-T781, Sanyo Electric Co., Tokyo, Japan) for the same periods as for the other treatments. All treatments and the corresponding controls were run in four repetitions.

2.3. Determination of DMSO concentration in eggs and embryos

After cryoprotectant impregnation, 5–8 unfertilized eggs or intact embryos from each of the treatment and control groups were rinsed with 0.15 M saline on a gauze and homogenized with 5 ml of 5% $HCLO_4$ (Wako Pure Chemical Industries Ltd., Tokyo, Japan) for protein precipitation [31]. The homogenate was passed through a 0.45 μm membrane filter (Toyo Roshi Kaisha Ltd., Tokyo, Japan) and a 10 μl aliquot was injected into an HPLC system as follows. The HPLC system (Shimadzu Corp., Kyoto, Japan) consisted of a Bio-Rad Aminex HPX-87H column (7.8 mm × 300 mm), Shimadzu LC-6A pump, CTO-6A column oven at 35 °C, SPD-6A UV detector at 210 nm, and Chromatopac C-R6A unit. The mobile phase was 0.003 M H_2SO_4 and the flow rate was set at 0.6 ml/min. The mean diameter of unfertilized eggs (1.1 mm) and embryos (1.4 mm) was measured under a microscope before treatment and used for calculation of the sample volume and the internal concentration of DMSO (C_{in}, mol per volume). These calculations assumed the

eggs to be spherical and no allowance was given for shrinkage or swelling during impregnation.

2.4. Fertilization and survival rate estimation

Estimation of the viability of unfertilized eggs after treatment was carried out by insemination of groups of 8–15 eggs with pooled milt from three males. Milt was collected from males anaesthetized with 0.1% 2-phenoxyethanol (Wako Pure Chemical Industries Ltd., Tokyo, Japan) using a 2 μl microtip syringe. Inseminated eggs were then activated and kept in fresh water for further development. Fertilization rate was scored 6 h after activation and normally developing embryos were selected for determination of survival as follows. Survival rates were determined in all treatments at the time of hatching (stage 39; 28). For this purpose, groups of 8–15 eggs were briefly rinsed in fresh water after the treatments and transferred to 10 l plastic tanks with fresh water at 25 °C for rearing until observation. Embryos were also assessed for the following developmental features: heart, eye, otolith, gall bladder and tail musculature development as well as cardiac movement.

2.5. Statistical analyses

The significance of the differences in the concentration of DMSO in eggs and embryos between the treatments and the corresponding controls was analyzed by the Student's *t*-test. Fertilization rates and survival rates were compared with the Yates' continuity corrected chi-square test. A probability value of $P < 0.05$ was taken as statistically significant.

3. Results

The internal concentration of DMSO (C_{in}) and the viability of unfertilized eggs, 8-cell and eyed embryos that were impregnated with different concentrations of the cryoprotectant under normal (0 atm) and elevated (50 atm) hydrostatic pressure are shown in Figs. 2–4, respectively. With the exception of a transient decrease in the DMSO C_{in} at 3–5 min of impregnation, which was observed in all experimental groups including those osmotically and thermally manipulated, different developmental stages showed characteristic patterns of pressure effectiveness, cryoprotectant uptake and tolerance.

Spontaneous activation was observed in 5–10% of the unfertilized eggs in all groups regardless of cryoprotectant concentration. In these unfertilized eggs, the DMSO C_{in} increased and egg viability decreased continuously until 20 min (Fig. 2). Cryoprotectant uptake, fertilization and survival rates were similar between the 0.6 and 1.2 M groups but showed proportional changes with higher concentrations of cryoprotectant. Unfertilized eggs impregnated at 50 atm took up considerably more DMSO than those at 0 atm but also showed a much faster decrease in fertilization rates than the controls. Thus, a significant proportion (30–50%) of the eggs in the cryoprotectant solution at 0 atm could still be fertilized after 20 min whereas at 50 atm all eggs lost fertility after only 10 min. Most or all (90–100%) embryos that were obtained by artificial insemination after impregnation

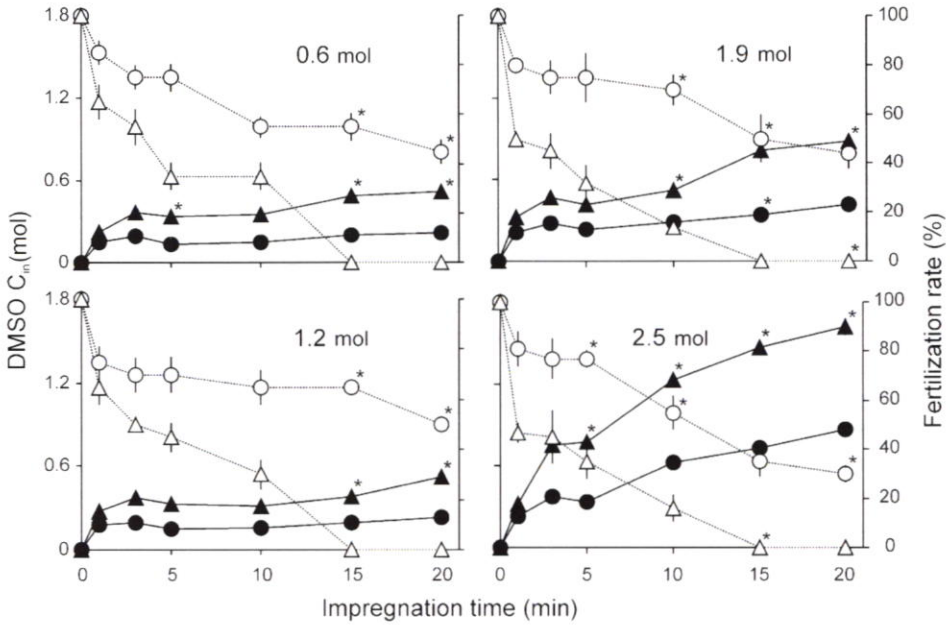

Fig. 2. Changes in the internal concentration of DMSO (DMSO C_{in}; closed symbols) and fertilization rates (open symbols) of medaka eggs exposed to 0.6–2.5 M cryoprotectant concentrations for different lengths of time under normal (0 atm; circles) and elevated (50 atm; triangles) hydrostatic pressure. Data shown as mean ± S.E.M. (vertical bars); asterisks indicate statistically significant differences between corresponding values ($P < 0.05$). Note: subsequent survival rates were between 90 and 100% of the fertilization rates for all groups.

developed and hatched normally regardless of cryoprotectant concentration and hydrostatic pressure.

In the 8-cell embryos, DMSO C_{in} peaked at 10–15 min in all groups and then decreased slightly at 20 min (Fig. 3). Survival rates, on the other hand, decreased 30–40% initially but the rate of decrease leveled off thereafter. As observed for unfertilized eggs, cryoprotectant uptake was very similar between the 0.6 and 1.2 M groups and proportionally elevated in the 1.9 and 2.5 M groups. Embryos impregnated at 50 atm took up about 15–40% more DMSO than those at 0 atm. The survival rates were not affected by hydrostatic pressure with the exception of the groups impregnated with 2.5 M DMSO at 50 atm for 15 and 20 min, which had slightly lower survival rates than the controls.

In the eyed embryos, cryoprotectant uptake and survival rates were very similar in all groups regardless of the concentration of the DMSO in the impregnation medium (Fig. 4). The DMSO C_{in} stabilized after 5 min of impregnation but survival rates decreased slightly until 20 min. Hydrostatic pressure had no measurable effect on the DMSO C_{in} but survival rates at 50 atm were slightly lower than those of controls at 0 atm, in particular after 10–15 min in the 1.9 and 2.5 M DMSO solutions.

Osmotic manipulation of 8-cell embryos prior to impregnation enhanced cryoprotectant uptake by 20–40% in comparison to untreated controls without any significant effect on survival rates (Fig. 5). No significant differences were observed between groups treated for

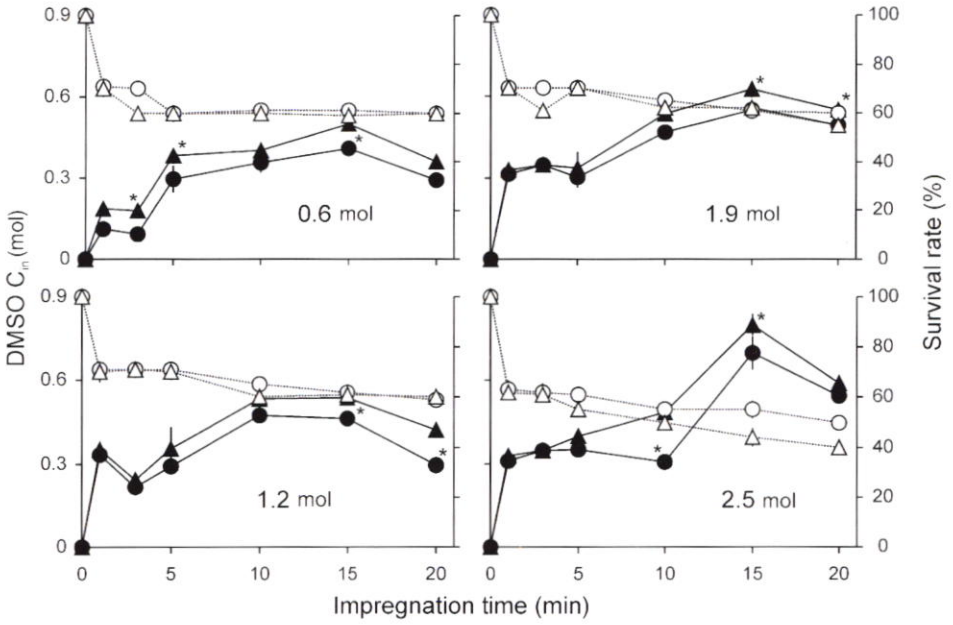

Fig. 3. Changes in the internal concentration of DMSO (DMSO C_{in}; closed symbols) and survival rates (open symbols) of 8-cell medaka embryos exposed to 0.6–2.5 M cryoprotectant concentrations for different lengths of time under normal (0 atm; circles) and elevated (50 atm; triangles) hydrostatic pressure. Data shown as mean ± S.E.M. (vertical bars); asterisks indicate statistically significant differences between corresponding values ($P < 0.05$).

1 and 3 min in trehalose (results for 1 min not shown). Cryoprotectant impregnation and survival rates varied in direct and inverse proportion with the temperature of impregnation, respectively (Fig. 6). The DMSO C_{in} of embryos at 20 °C, for instance, was more than double that of embryos impregnated at 0 °C; survival rates, in turn, were about 40% less.

4. Discussion

The results of this study are relevant to the discussion of the suitability of eggs and embryos for cryopreservation. The use of unfertilized eggs [18] or of inseminated but nonactivated eggs [32] has been proposed as a possible means to overcome one of the major barriers to cryoprotectant penetration, the perivitelline membrane [11,33]. Also, because the chorion of fish eggs hardens upon activation, it is generally assumed that embryos are less permeable to cryoprotectants than unfertilized eggs. However, even though eggs are undoubtedly structurally simpler than embryos, it is not a straightforward conclusion that they are more suited for cryopreservation. In fact, few studies have actually measured the permeability of fish eggs and intact embryos to cryoprotectants [13,34]. The results of this study, in which the internal concentration of DMSO was directly measured by HPLC, clearly indicate that the initial permeability, as shown by the DMSO C_{in} at 1–5 min, is

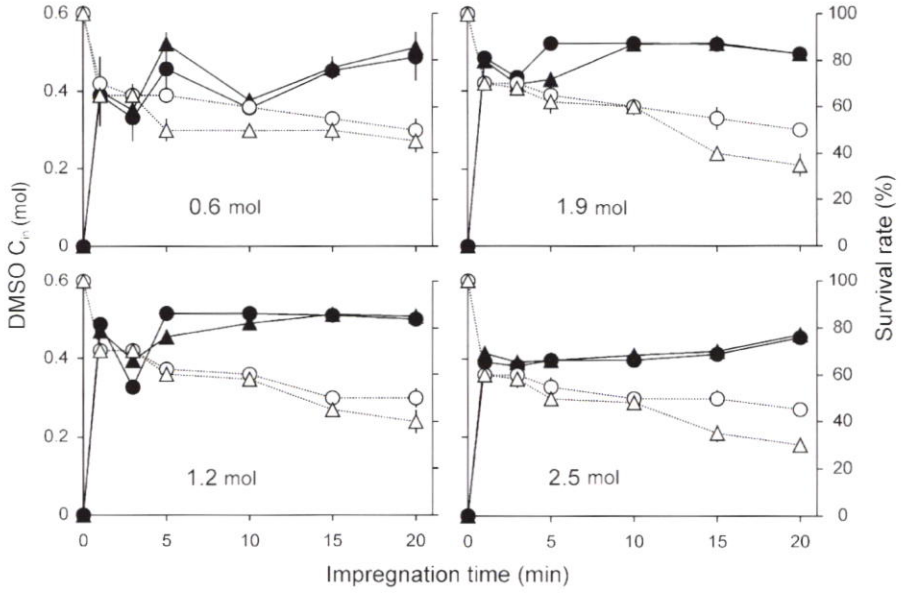

Fig. 4. Changes in the internal concentration of DMSO (DMSO C_{in}; closed symbols) and survival rates (open symbols) of eyed embryos of medaka exposed to 0.6–2.5 M cryoprotectant concentrations for different lengths of time under normal (0 atm; circles) and elevated (50 atm; triangles) hydrostatic pressure. Data shown as mean ± S.E.M. (vertical bars); asterisks indicate statistically significant differences between corresponding values ($P < 0.05$).

higher in embryos than in eggs and that it increases with further embryonic development. The absolute uptake after longer impregnation (10–20 min), on the other hand, was highest in unfertilized eggs followed by 8-cell embryos and then eyed embryos. Our results cannot explain whether this paradoxical inversion in the DMSO uptake with time represents an actual change in permeability rates, or if it is due to cryoprotectant-induced damage to the

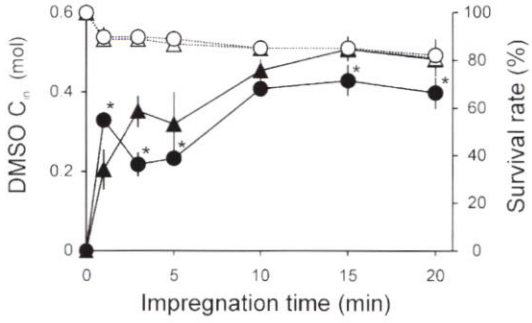

Fig. 5. Changes in the internal concentration of DMSO (DMSO C_{in}; closed symbols) and survival rates (open symbols) of 8-cell medaka embryos that were exposed to 1 M trehalose solution for 3 min (triangles) or not (controls; circles) and subsequently impregnated with 1.2 M DMSO for different lengths of time. Data shown as mean ± S.E.M. (vertical bars); asterisks indicate statistically significant differences between corresponding values ($P < 0.05$).

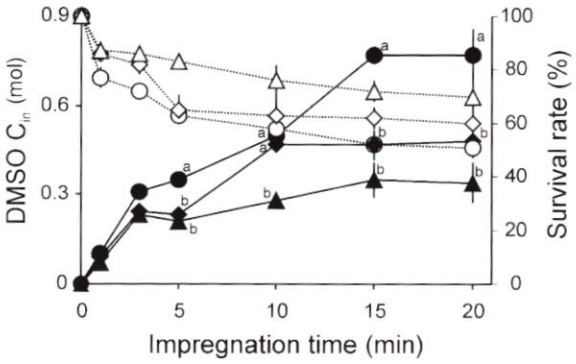

Fig. 6. Changes in the internal concentration of DMSO (DMSO C_{in}; closed symbols) and survival rates (open symbols) of medaka eyed embryos impregnated with 1.5 M DMSO for different lengths of time at 0 (triangles), 5 (diamonds) and 20 °C (circles). Data shown as mean ± S.E.M. (vertical bars); corresponding values with different letters are significantly different ($P < 0.05$).

eggs and younger embryos. Younger stages are generally less tolerant to cryoprotectants than older ones [20,21,35] and the passive uptake of cryoprotectant could be enhanced in dead cells or through damaged membrane sites. Also, this phenomenon is unlikely to be due to spontaneous activation of the unfertilized eggs [29] since in our experiments less than 10% of the eggs were activated in the DMSO-YR solution. Another interesting finding was that the DMSO C_{in} was remarkably constant after 1–5 min in eyed embryos but not in 8-cell embryos, which also possess a well-developed perivitelline space, suggesting that the former are somehow able to block additional uptake of DMSO or excrete its excess. Further studies must clarify whether this phenomenon is related to the development of ionic and osmotic regulation, which likely begins prior to epiboly [36], or excretory ability. In any case, this could represent an additional barrier to impregnation of older embryos with cryoprotectant. On a DMSO C_{in} basis, however, the viability rates for the eggs and two stages of embryos are not much different. Thus, different types of materials appear to have both advantages and disadvantages, and their suitability must be considered in relation to their chilling and handling (mechanical) tolerances, the possibility of enhancing impregnation rates by alteration of the physical, chemical, thermal or osmotic environment, and possibly the results of cryopreservation itself.

A second important consideration is the selection of the proper C_{in} for cryopreservation and the method to attain it. The C_{in} required for cryopreservation is not known but intuitively it should be as high as possible so long as survival rates can be maintained at satisfactory levels. Thus, a basic question arises as to whether high or low concentrations of cryoprotectant, long or short impregnation times, and single-step or stepwise treatments are the most adequate. This study did not investigate stepwise impregnation, as it seems to confer no significant advantage for embryos of fish [9] and domestic animals [37]. As can be seen in this study and in Suzuki et al. [19], mortality is largely a function of the DMSO C_{in} rather than that of the concentration of the impregnating medium. This would imply that the concentration of the medium and the duration of impregnation are not relevant. However, careful inspection of the data revealed that, regardless of developmental stage,

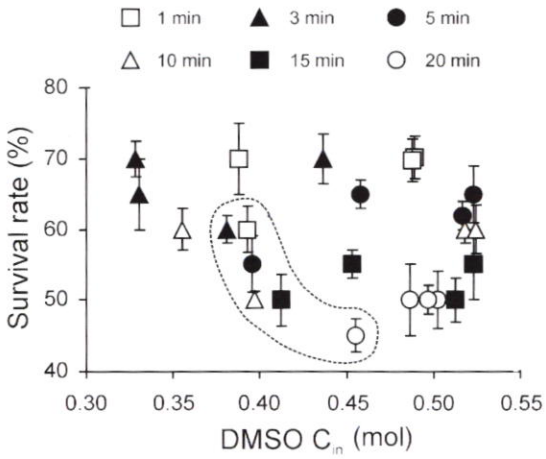

Fig. 7. Survival of eyed embryos of medaka as a function of the internal concentration of DMSO (DMSO C_{in}), impregnation time, and the concentration of cryoprotectant (based on results from Fig. 4 values for 2.5 M are encircled by a dotted line). It can be seen that survival rates for any given DMSO C_{in} are inversely related to the impregnation time. Data shown as mean ± S.E.M. (vertical bars) of 3–4 trials for each set of conditions.

the highest survival rates for any given C_{in} were generally obtained with the shortest impregnation times (Fig. 7). The example shown in Fig. 7 also reveals that impregnation in 2.5 M DMSO substantially reduced survival in comparison to the other medium concentrations for any given impregnation time even though the DMSO C_{in} achieved is sometimes less than that with the other concentrations. This result supports the observation by other authors that the survival of medaka embryos decreases dramatically above 2 M DMSO [19,38,39] and suggests that this decrease is not only due to DMSO C_{in}-induced toxicity. One possibility is the initial dehydration and change in volume caused by the high cryoprotectant concentration [16]. Thus, this study indicates that faster impregnation times are more conducive to higher prefreezing survival rates but these might not be attainable simply by further increasing the concentration of the impregnation medium. Also, if stepwise impregnation is attempted, it must consist of very short steps to avoid prolonged exposure to the cryoprotectant.

As seen above, it is important to find methods to accelerate cryoprotectant uptake that do not rely on increasing the medium concentration. Relatively few studies have been conducted on the effects of positive hydrostatic pressure on the permeability of biological membranes. In a study using human red blood cells, Hall et al. [40] noted that the K^+ permeability of the cells increased under pressure. In aquatic animals, Roer et al. [41] demonstrated changes in the Na^+ flux in the cells of freshwater gammarid amphipods subjected to hydrostatic pressures of 60–140 atm whereas Serbert et al. [42] showed that a pressure of 101 atm enhanced by 1.6 times the glycolytic flux in the white muscle of freshwater eel, *Anguilla anguilla*. However, there are no reports available on the behavior of fish eggs and embryos during impregnation with cryoprotectants under elevated hydrostatic pressure. The results of this study showed that the DMSO C_{in} in 8-cell embryos impregnated at 50 atm was consistently higher that those in control embryos

at 0 atm. An even greater increase in the DMSO uptake under pressure was observed in unfertilized eggs, although part of this increase could be the result of damage to the eggs, as discussed previously. Nevertheless, short-term (3–5 min) impregnation of unfertilized eggs under 50 atm produced results as good as those of groups treated for 20 min at 0 atm. Interestingly, the same level of hydrostatic pressure had no significant effect on the uptake of cryoprotectant in eyed embryos. These results suggest marked stage-dependent differences in pressure tolerance and its effectiveness to promote cryoprotectant uptake.

The osmotic conditions of the eggs and embryos and the temperature of impregnation also had a significant effect on the uptake of cryoprotectant although only one developmental stage (8-cell embryo) was examined. Exposure of these embryos to trehalose (1 M), a disaccharide containing two glucose molecules, prior to impregnation with DMSO enhanced the uptake of the cryoprotectant by 20–40%. The rationale of this approach was to partially dehydrate the embryos in trehalose, which has low permeation rates across biological membranes, in order to facilitate the uptake of the waterborne DMSO during impregnation. Nonpermeating agents are used sometimes to dehydrate cells during impregnation or to help remove cryoprotectant after thawing [43] but this appears to be the first report on the use of partial dehydration to enhance subsequent cryoprotectant uptake. More importantly, osmotic manipulation with trehalose had no adverse effect on the survival rates, as has been seen in other studies [8]. These findings were recently confirmed in additional experiments with this and another species, the pejerrey *Odontesthes bonariensis* (Routray, unpublished observations). The present study also showed that the uptake of DMSO by medaka embryos was directly proportional to the temperature of impregnation, as has been observed in mammalian gametes and embryos. Mouse and human oocytes, for example, took up more DMSO at 30 °C than at 0 or −3 °C [26] and 10 °C [44], respectively. This enhanced uptake was probably related to the progressive DMSO-induced destabilization of the phospholipid bilayer at higher temperatures [27]. On the other hand, DMSO toxicity for the 8-cell embryos treated at different temperatures also increased with increasing temperature as seen in many other biological systems [35]. Thus, it is also possible that part of the enhanced uptake might have been due to cryoprotectant-induced damage to the cells, as previously discussed.

The results of this study greatly extend the observations by other authors on factors such as cryoprotectant concentration, impregnation time and the developmental stage (or type) of the materials and their interactions, and provide evidence that hydrostatic pressure and temperature during impregnation, as well as the osmotic conditions of the materials, can be manipulated to increase the uptake of cryoprotectant by fish eggs and embryos. In the example of 8-cell embryos, hydrostatic pressure or osmotic manipulation can enhance the uptake of DMSO by as much as 40%. Temperature also had a marked effect on the uptake of the cryoprotectant. Thus, although the individual contribution of these factors is not as large as that reported for some other factors (e.g. the several-fold enhancement with ultrasound treatment [21]), they may be complementary to other strategies and hence be critical for the optimization of impregnation protocols. In fact, related studies have shown that cryoprotectant impregnation by these methods significantly reduced the nucleation temperature of medaka embryos [45]. Nevertheless, studies must be conducted to elucidate the mechanisms involved in the modification of DMSO uptake by these factors and to quantify the relative fractions of cryoprotectant in the perivitelline space and in the

embryonic tissue proper. Also, further research should investigate other levels and conditions for hydrostatic, thermal, and osmotic treatment of eggs and embryos during cryoprotectant impregnation, their interactions, and whether these findings also apply to other types of cryoprotectants and fish species.

Acknowledgements

We thank Dr. T. Shirai, Department of Food Science and Technology, for kind assistance with DMSO measurements and Dr. T. Hagiwara for technical assistance during the course of this study. This study was partly supported by a scholarship to Routray and a grant to C.A. Strüssmann from the Ministry of Education, Culture, Sports, Science and Technology, Japan (grant no. 12460085).

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