# Factors affecting the uptake of cryoprotective agents by fish eggs and embryos

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## INTRODUCTION

Cryopreservation of fish eggs and embryos has not been accomplished yet and one of the major reasons seems to be the insufficient impregnation with cryoprotective agents.[1-3] The complex structure of these materials, including their large size, thick chorion, membrane-bound compartmentalization, and large amount of yolk, is an obstacle to the uptake of permeating cryoprotectants such as dimethyl sulphoxide (DMSO).[4] Likewise, little is known about the biological and physical factors that affect membrane permeability in fish eggs and embryos. In this study, we evaluated the suitability of eggs and two embryo stages for cryoprotectant impregnation and the feasibility of manipulating the temperature, hydrostatic pressure, and the osmotic conditions during impregnation to promote the uptake of DMSO by fish eggs and embryos.

## MATERIALS AND METHODS

Eggs and embryos of medaka (*Oryzias latipes*) and pejerrey (*Odontesthes bonariensis*), two freshwater species that spawn relatively large demersal eggs, were used in this study. Unfertilized eggs were collected by gentle stripping the abdomen (pejerrey) or by dissection (medaka). Embryos were obtained by allowing the females to spawn naturally in the presence of males. After collection, normally developing embryos were selected and incubated until the desired stage. To compare the uptake of DMSO by the different materials, 10-30 unfertilized eggs, 8-cell (2 hr) and eyed (5-6 days) embryos of medaka were immersed for 1, 3, 5, 10, 15 and 20 min in a 1.2M DMSO solution in Yamamoto ringer. After impregnation, the internal DMSO concentration ($C_{in}$) of 5-8 eggs or embryos per group was analyzed by high performance liquid chromatography (HPLC) following the methods of Suzuki et al.[9] The remaining eggs and embryos were used for viability estimation. Unfertilized eggs were inseminated and viability was scored 6 hr after activation in fresh water. Survival of embryos was estimated after incubation for 10-11 days. The same protocols were used to investigate the effects of the temperature, hydrostatic pressure, and osmotic conditions during impregnation on cryoprotectant uptake. The influence of temperature was examined by impregnating 8-cell pejerrey embryos in 1.5M DMSO at temperatures of 0, 5, and 20°C. Unfertilized eggs and eyed embryos of medaka were impregnated with 2.5M DMSO in a pressure vessel under 0 and 50 atm (at 20°C) to investigate the effect of hydrostatic pressure. For osmotic manipulation, 8-cell pejerrey embryos were first partially dehydrated for 1 or 3 min in a 1M solution of the low permeating disaccharide trehalose and then impregnated with 1.2M DMSO. Each experiment was repeated 3-4 times. The significance of the differences in $C_{in}$ between experimental and controls groups was analyzed by ANOVA. Fertilization/survival rates were compared with the Yates' continuity corrected Chi-square test.

## RESULTS

The initial uptake rate and maximum DMSO $C_{in}$ were higher in medaka embryos than in eggs and increased with embryonic development (Fig. 1A). However, the $C_{in}$ of eyed embryos remained constant after 5 min. Viability rates were approximately similar in the three groups. The uptake of DMSO and viability of 8-cell pejerrey embryos were directly and inversely proportional to the impregnation temperature, respectively (Fig. 1B). Elevated hydrostatic pressure promoted DMSO uptake but caused rapid loss of viability in unfertilized eggs of medaka; in contrast, it had no measurable effect on eyed embryos (Fig. 1C). Partial dehydration of 8-cell pejerrey embryos in trehalose solution prior to impregnation significantly increased the DMSO $C_{in}$ in comparison to untreated controls without any significant effect on the survival rates (Fig. 1D).

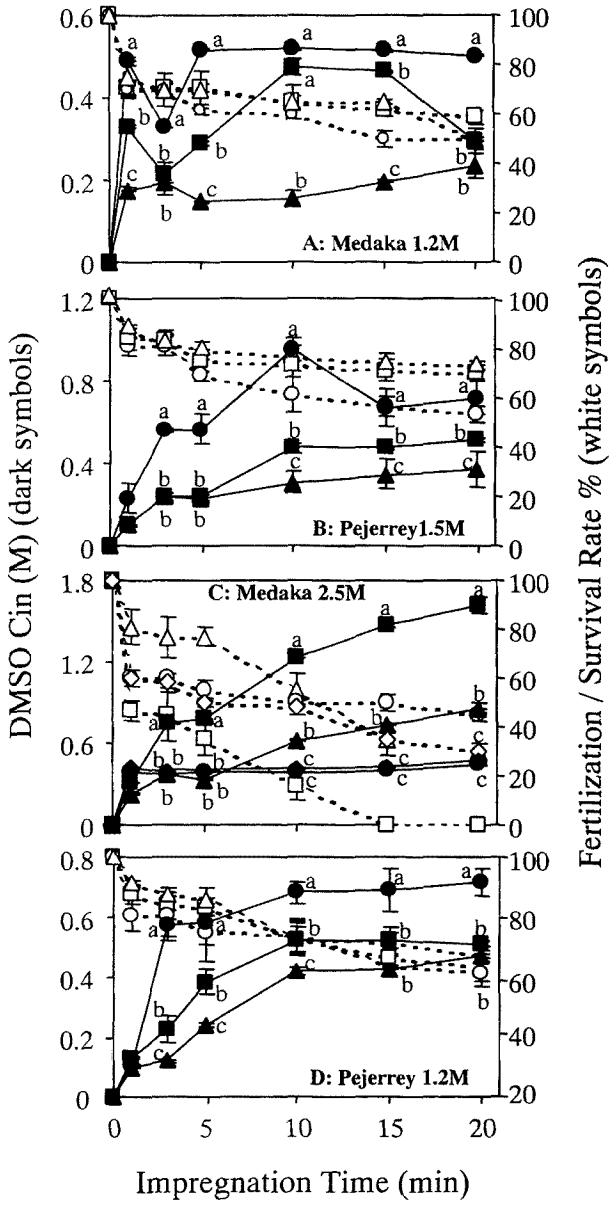


**Fig. 1** Effects of various factors on the uptake of DMSO ($C_{in}$; dark symbols) and fertilization/survival rates (white symbols) of fish eggs and embryos. The species and DMSO concentration during impregnation are shown in the panels. Data shown as mean ± SE; corresponding values with different letters vary significantly ($P<0.05$). **A**: Egg or embryo stages; ▲, unfertilized eggs; ■, 8-cell embryos; ●, eyed embryos. **B**: Temperature; ▲, 0°C; ■, 5°C; ●, 20°C. **C**: Hydrostatic pressure; ◆, eyed embryo, 0 atm; ●, eyed embryo, 50 atm; ▲, unfertilized egg, 0 atm; ■, unfertilized egg, 50 atm; **D**: Osmotic conditions; ▲, control; ■, 1 min dehydration; ●, 3 min dehydration

## DISCUSSION

This study showed that unfertilized eggs took up less cryoprotectant than embryos during impregnation with 1.2M DMSO (at 0 atm), even though the former are structurally simpler than the latter. However, this trend was reversed under a DMSO concentration of 2.5M and even more so at 50 atm. The exact reason for this difference in uptake between eggs and embryos according to the impregnation concentration is unknown but it could be related to a lower tolerance of the eggs to DMSO[4] and the passive entry of cryoprotectant due to damaged membrane sites and non-viable cells. Differential tolerance and loss of viability could be responsible also for the dramatic increase in DMSO uptake in the eggs under 50 atm. However, eggs impregnated under 50 atm for 3-5 min showed higher DMSO $C_{in}$ and survival rates than those kept for periods as long as 20 min but at 0 atm. In 8-cell embryos, elevated hydrostatic pressure improved DMSO uptake without loss of viability (P. Routray et al., unpublished results). Thus, from the point-of-view of impregnation, different stages seem to differ in suitability for cryopreservation. Also, hydrostatic pressure seems to alter membrane permeability in fish eggs and embryos but its effects are stage- and probably also concentration-dependent. The other examined factors, impregnation temperature and osmotic manipulation, had a clear effect on the uptake of DMSO. The proportional uptake of DMSO with temperature by 8-cell embryos resembles the improved uptake by mouse and human embryos at 30°C in comparison to 0 or -3°C.[9] As expected, partial dehydration of the embryos prior to impregnation greatly enhanced the uptake of DMSO. It was assumed that an osmotic differential caused by dehydration might facilitate the uptake of waterborne DMSO during impregnation. Thus, the results of this study indicate that hydrostatic pressure, temperature of impregnation and the osmotic conditions of the materials can be manipulated to increase the uptake of cryoprotectant and perhaps lead to successful cryopreservation of fish eggs and embryos.

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