Theriogenology 79 (2013) 853–858

Contents lists available at SciVerse ScienceDirect

Theriogenology

journal homepage: www.theriojournal.com

Electroporation enhances permeation of cryoprotectant (dimethyl sulfoxide) into Japanese whiting (*Sillago japonica*) embryos

Sk.M. Rahman, C.A. Strüssmann*, T. Suzuki, M. Watanabe

Graduate School of Marine Science and Technology, Tokyo University of Marine Science and Technology, Minato-Ku, Tokyo, Japan

ARTICLE INFO

Article history:
Received 31 August 2012
Received in revised form 2 January 2013
Accepted 3 January 2013

Keywords:
Cryopreservation
Whiting embryos
DMSO
Electroporation
Permeation

ABSTRACT

Survival after cryopreservation has never been achieved with fish embryos, presumably because of insufficient cryoprotectant permeation before cooling. The objective was to determine the relative efficiency of electroporation for incorporation of cryoprotectant into Japanese whiting embryos and survival of electroporated embryos in DMSO after freeze-thawing. Embryos (somites stage) subjected to electroporation at 100, 200, or 300 V in artificial sea water had similar hatching rates (94%–96%) as untreated control embryos (0 V; 97%) and those treated with voltages between 400 and 900 had survival rates of 88% to 0%. Embryos (somites stage) electroporated at 300 V in 10%, 20%, or 30% DMSO/artificial sea water solutions had hatching rates of 94%, 88%, and 85%, respectively, and DMSO contents of 10, 30, and 78 mM. Embryos treated with higher voltages had higher DMSO uptake (up to 84 mM), but reduced survival (62%–6%). Pre-exposure of embryos to 10% DMSO for 20 minutes before electroporation improved DMSO uptake (116 mM). Embryos treated with DMSO and electroporated under the best conditions determined in this study did not resume development after attempted vitrification. We concluded that electroporation enhanced DMSO uptake by fish embryos, but concentrations obtained with this procedure alone were apparently insufficient to prevent internal ice formation during cooling and thawing.

1. Introduction

Sperm, egg, and embryo cryopreservation are desirable tools for conservation of commercially important and endangered fish species, ensuring a stable supply for the aquaculture industry, and storage of genetic materials for future study and use. Cryopreservation of fish sperm is well established and fish gene banks (based on sperm) are in operation in several countries. Conversely, although tremendous efforts have been undertaken in the past decades, there has been limited progress in cryopreservation of fish eggs and embryos. This failure has been attributed to insufficient cryoprotectant (CPA) permeation before cooling [1–4]. In that regard, the large size of fish embryos results in a low surface area to volume ratio and reduces the total amount of CPA that can be taken up [5,6]. Consequently, insufficient internal CPA concentrations cause physical damage to embryos, because of formation of intra- and extracellular ice crystals, particularly during slow cooling and thawing [7–9]. Vitrification, which can be achieved by ultrarapid cooling of biological materials in combination with high internal concentrations of CPAs, might overcome ice crystal formation. However, for this purpose, innovative methods must be developed to achieve fast and uniform permeation of the CPA into the various compartments of fish embryos while concurrently minimizing CPA toxicity.

Membrane electropermeabilization (or electroporation) is useful for introduction of membrane-impermeable xenomolecules (e.g., drugs, hormones, proteins, plasmids, etc.) into living cells, and the controlled release of intracellular

* Corresponding author. Tel.: +81 3 5463 0541; fax: +81 3 5463 0541.
E-mail address: carlos@kaiyodai.ac.jp (C.A. Strüssmann).

0093-691X/$ – see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.theriogenology.2013.01.002

substances [10,11]. Electropermeabilization is based on the temporary increase of membrane permeability caused by reversible electrical breakdown of the plasma membrane on application of external high-intensity but very short duration field pulses [12]. Because this approach was used to incorporate trehalose into mouse myeloma cells (Sp2 line) for cryopreservation [13], perhaps electroporation could improve the rate of CPA permeation into fish embryos. However, this has apparently never been reported.

The objective of the present study was to determine the relative efficiency of electroporation for impregnation of CPA into embryos of Japanese whiting (*Sillago japonica*), a model marine fish used in our previous studies of fish embryo cryopreservation [14,15]. Thus, we determined the effects of electroporation conditions (voltage, number of pulses, pre- and posttreatment exposure to CPA, and CPA concentrations) on embryo survival and the internal concentrations of CPA in embryos. Dimethyl sulfoxide (DMSO) was chosen as the cryoprotectant for this study, because it is relatively nontoxic to whiting embryos compared with other CPAs [15] and because its concentration in embryos can be accurately measured by high performance liquid chromatography [16]. In addition, we also compared the suitability of two embryonic developmental stages (somites and tail elongation stages) for electroporation-mediated CPA impregnation and performed a preliminary cryopreservation trial using the best conditions determined in this study.

2. Materials and methods

2.1. Embryo collection and handling procedures

Sexually mature Japanese whiting were collected from a wild population in Tateyama Bay, Japan, and reared in 1200 L recirculated water tanks under a controlled day-night cycle (14 hours light/10 hours dark, at 25 °C). Experiments were performed according to the guidelines for animal care and handling of Tokyo University of Marine Science and Technology, Tokyo, Japan. Water salinity was adjusted to 33 psu (osmolality of 991 mmol/kg) with dechlorinated tap water and artificial sea water (ASW) salts (Rei-Sea Salt G, Tokyo, Japan) and the animals were fed frozen krill twice daily. Female and male fish were kept together at the ratio of 1:1 and spawnings were observed nearly every day. The fertilized, buoyant eggs were collected with a net and incubated in 500 mL ASW with mild aeration at 25 °C until the desired developmental stage. Two developmental stages, e.g., somites (14–16 somites; approximately 14 hours after fertilization) and tail elongation (23–24 somites; approximately 16 hours after fertilization) [17] were used in this study.

2.2. Electroporation conditions and experimental trials

Four experiments were conducted to determine the best conditions of electroporation for incorporation of DMSO into whiting embryos. Electroporation was performed using a transfection chamber (Shimadzu FTC-64, electrode distance 4 mm; Shimadzu, Kyoto, Japan) and gene transfer equipment (Shimadzu GTE-10). The pulse length and capacitance were 500 μs and 1 μF, respectively. Embryos were loaded in the electroporation chamber using 0.5 mL of ASW (adjusted to 33 psu with distilled water) or DMSO (Wako, Osaka, Japan) solutions prepared with ASW. Unless otherwise specified, only one pulse was applied. Each treatment was applied to five replicate batches of 50 embryos for assessment of survival and to three replicate batches of 30 embryos for measurement of DMSO uptake. Embryos not subjected to electric pulse (0 V) or exposed to DMSO were considered control groups (depending on the experiment). The entire electroporation procedure (loading into the chamber, application of the electric pulse, and transfer to clean water or collection for DMSO measurement) was completed in approximately 1 minute; therefore, nonelectroporated controls were subjected to 1 minute of DMSO impregnation. After each treatment, embryos were rinsed in ASW for 10 minutes and incubated in plastic Petri dishes containing 5 mL of fresh ASW at 25 °C to monitor hatching and survival for intervals up to 24 hours. Hatching, which usually occurs 20–22 h after fertilization at 25 °C, was considered an indication of embryo viability.

In the first experiment, the effect of electroporation voltage on survival of whiting embryos in ASW was determined. Embryos at the somites stage were electroporated at 0 (control), 100, 200, 300, 400, 500, 600, 700, 800, or 900 V.

The second experiment examined the effects of electroporation at 0 (control), 300, 400, or 500 V in DMSO (10%, 20%, or 30%) on survival and uptake of DMSO of embryos at two developmental stages (somites and tail elongation). The choice of voltages was based on the results of the previous experiment; in that study, hatching rates of somites-stage embryos electropored in ASW decreased significantly (compared with the untreated control) at voltages greater than 400 V (see Fig. 1).

The third experiment examined the effects of pre- and postelectroporation exposure to DMSO on survival and uptake of DMSO by somites-stage embryos. For pretreatment, embryos were immersed in 10% DMSO for 20 minutes at room temperature before electroporation at 300 V in 30% DMSO. For posttreatment, embryos were electroporated at 300 V in 30% DMSO and left in the same solution for additional intervals of 3, 6, and 9 minutes.

The fourth experiment was designed to evaluate whether multiple (periodic) electric pulses could promote further DMSO uptake. In this trial, somites-stage embryos were subjected to 1, 2, or 3 pulses of 300 V at 10-second intervals in 0% or 30% DMSO solutions.

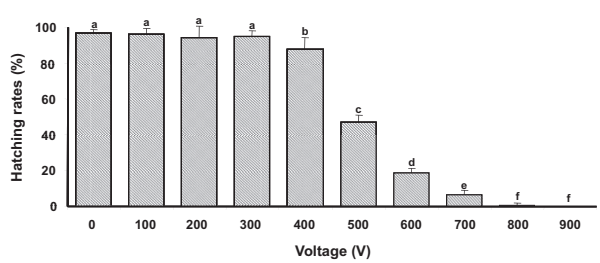

Fig. 1. Mean ± SD hatching rates of whiting (*S. japonica*) embryos (somites-stage) after electroporation in artificial sea water with various voltages. Means without a common letter (a–f) differed ($P < 0.05$).

2.3. Analysis of DMSO content of embryos

Concentration of DMSO in embryos after electroporation and exposure to the CPA was measured by high performance liquid chromatography. Measurements were performed as described [16]. The analytical system and operating conditions included a Bio-Rad Aminex HPX-87 column (7.8 mm × 300 mm; Shimadzu, Kyoto, Japan), Shimadzu LC-6A pump, CTO column oven (at 35 °C), SPD-6A detector (at 210 nm), and a Chromatopac C-R6A unit. The mean diameter of the embryos (0.68 mm) was measured using a microscope before treatment and used for calculation of their volume and the internal concentration of DMSO.

2.4. Cryopreservation trial

Embryos at the somites stage were used to test the effectiveness of electroporation-mediated CPA impregnation for actual cryopreservation. Embryos were exposed to a single 300 V electric pulse in 30% DMSO solution, with or without pretreatment with 10% DMSO solution for 20 minutes before electroporation in 30% DMSO solution. Electroporated embryos were then loaded in 0.25-mL straws and plunged directly into LN_2 in a 2 L Dewar flask for 40 to 60 minutes. Straws were subsequently removed from LN_2, thawed for 5 seconds at 40 °C, and their contents transferred to clean ASW at 25 °C for observation of embryo morphology and survival. Conditions for thawing were determined empirically to minimize the probability of recrystallization during thawing. Each treatment consisted of seven replicates with approximately 30 embryos each.

2.5. Statistical analyses

All numeric results are expressed as means ± SD. The statistical significance of differences among mean internal DMSO concentrations in embryos from various treatments was analyzed by one-way ANOVA, followed by the Tukey multiple comparison tests using GraphPad Prism 4 software (GraphPad Software, La Jolla, CA, USA). Differences with a P value of <0.05 were considered statistically significant.

3. Results

3.1. Survival and DMSO content of embryos after electroporation

Hatching rates of somites-stage embryos after electroporation in ASW at different voltages are summarized (Fig. 1). Embryos subjected to 100, 200, or 300 V had hatching rates similar to that of controls (94%–96%). Hatching rates decreased slightly at 400 V (88%) and markedly from 500 to 900 V (47%–0%).

Hatching rates of somites- and tail elongation-stage embryos after electroporation in various concentrations of DMSO at 300, 400, and 500 V are summarized (Fig. 2). Hatching rates were at least 85% and 77% for somites and tail elongation embryos, respectively, after electroporation in 10% to 30% DMSO solutions at 300 V. Survival decreased rapidly with increased DMSO concentration and electroporation voltage. In general, the somites stage was more tolerant to electroporation in DMSO solutions than the tail elongation stage. The uptake of DMSO by embryos increased with increasing concentration of the CPA and electroporation voltage (Fig. 3), although it could not be measured in some cases (30% DMSO at 400 and 500 V) because of massive embryo mortality. The highest internal CPA concentrations were recorded after electroporation in 20% DMSO at 500 V and 30% DMSO at 300 V in tail elongation-stage embryos. Somites-stage embryos generally had less internal DMSO than embryos at the tail elongation stage.

Embryos pre-exposed to 10% DMSO for 20 minutes before electroporation (300 V) in 30% DMSO had survival similar to electroporated, unexposed controls (Fig. 4). All embryos left in 30% DMSO after electroporation died within 6 minutes after treatment, whereas only 9% survived a postexposure of 3 minutes. Pre-exposure of embryos to 10% DMSO for 20 minutes enhanced internal content CPA concentration after electroporation compared with unexposed embryos (116 vs. 82 mM; Fig. 4). Post-electroporation exposure to DMSO caused heavy mortality and therefore was not considered further for measurement of internal DMSO content.

Hatching rates of embryos after multiple electric pulses in ASW were 89%, 90%, and 83% for one, two, and three pulses, respectively, whereas those in 30% DMSO decreased

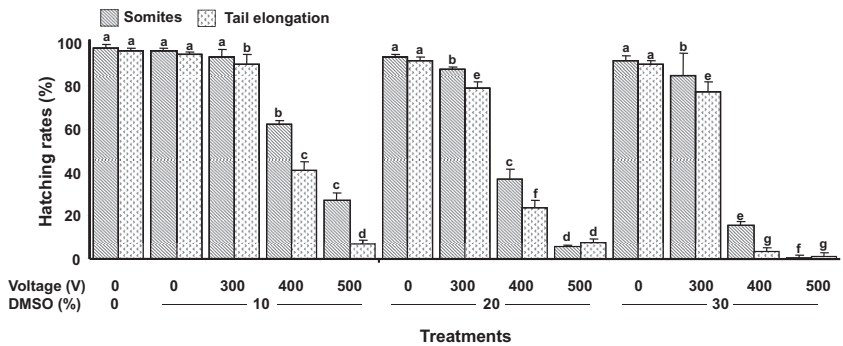

Fig. 2. Mean ± SD hatching rates of whiting (*S. japonica*) embryos (somites and tail elongation stages) after electroporation with various voltages and DMSO concentrations. Means without a common letter (a–g) differed ($P < 0.05$).

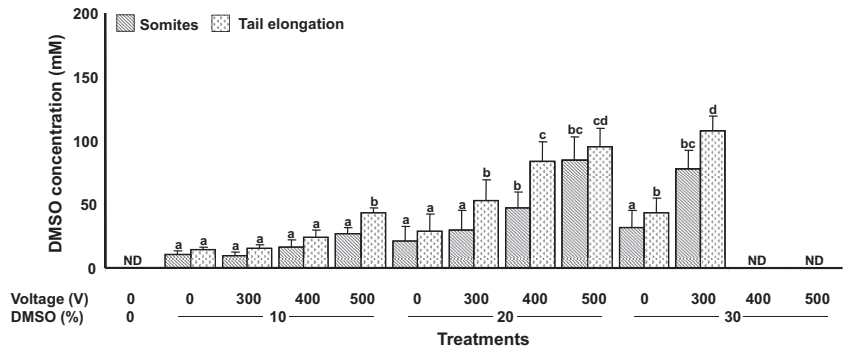

Fig. 3. Mean ± SD concentrations of DMSO in whiting (*S. japonica*) embryos (somites and tail elongation stages) after electroporation with various voltages and DMSO concentrations. Means without a common letter (a–d) differed ($P < 0.05$). ND, not determined.

gradually (58%–6%) with an increased number of pulses (Fig. 5). The uptake of DMSO was not measured in this trial, because it became evident that multiple pulses were highly lethal to embryos.

3.2. Cryopreservation trial

Embryos plunged into liquid nitrogen after electroporation-mediated CPA impregnation had varying appearances after freeze-thawing, ranging from an apparently intact morphology to evident damage including breakdown of the chorion and leakage of contents (results not shown). Proportions of intact embryos were not apparently different between batches pretreated with 10% DMSO and those not treated. However, even embryos with an apparently intact morphology did not have any developmental ability during incubation.

4. Discussion

Fish embryo cryopreservation is still not feasible either by slow, controlled cooling or by rapid cooling such as during vitrification. Insufficient impregnation of CPAs into fish embryos has been considered the major constraint to achieve successful cryopreservation [1–4]. To the best of our knowledge, this was the first report regarding electroporation as a tool to promote membrane permeability and cryoprotectant permeation in fish embryos.

Fish embryos tolerated electroporation voltages up to 400 V fairly well, whereas higher voltages were obviously detrimental. It was also evident from the hatching rates of embryos electroporated in ASW and DMSO solutions that mortality after electroporation was enhanced in the presence of CPA. Thus, high voltages were detrimental, probably also because they induced higher internal CPA concentrations in embryos, leading to CPA toxicity. For example, hatching rates of somites-stage embryos electroporated at 300 V were higher than 85% (>87% of the untreated controls) at all DMSO concentrations (0% to 30%) whereas those of embryos electroporated at 400 V dropped sharply from 88% (91% of the controls) at 0% DMSO to 15% (16% of the controls) at 30% DMSO. This conclusion was also supported by DMSO uptake. For example, internal CPA concentrations in embryos electroporated in 20% DMSO increased nearly three-fold between 0 and 500 V, and was associated with a drastic decrease in hatching rates (from 93% to 6%) in this range of voltages.

It is frequently suggested that cell viability decreased with increasing voltage and pulse length [18–20]; therefore, optimization of electroporation conditions is imperative for its successful application. Many embryos electroporated using voltages >300 V in both ASW and DMSO solutions

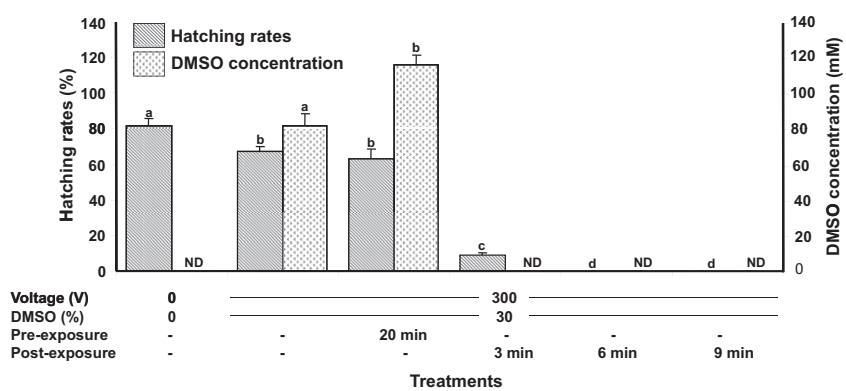

Fig. 4. Mean ± SD hatching rates and internal DMSO concentrations of whiting (*S. japonica*) embryos (somites-stage) exposed to DMSO solutions before (10%) or after (30%) electroporation in 30% DMSO. Means without a common letter (a–d) differed ($P < 0.05$). ND, not determined.

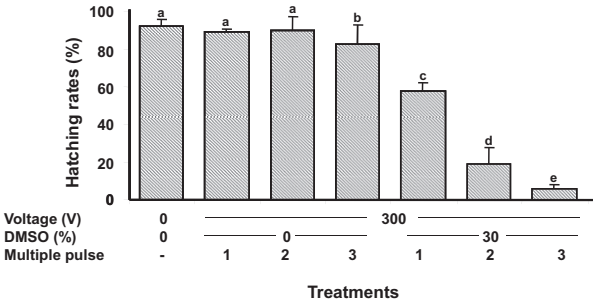

Fig. 5. Mean ± SD hatching rates of whiting (*S. japonica*) embryos (somites-stage) after electroporation (300 V) with single or multiple pulses at two DMSO concentrations. Means without a common letter (a–e) differed ($P < 0.05$).

began to sink within 10 minutes after the procedure, which is the first indication of loss of viability. In this study, embryos electroporated in 30% DMSO had reduced hatching rates when more than one pulse was applied. Similarly, additional exposure to 30% DMSO solution after an electric pulse in this solution caused marked loss of embryo viability. The loss of embryo viability caused by multiple pulses or prolonged exposure to DMSO solution could be because these conditions induce excessive DMSO uptake and toxicity, as discussed earlier in this article. Differences in survival between developmental stages were not as marked as those between voltages; regardless, embryos at the tail elongation stage were slightly less tolerant to the procedure than somites-stage embryos, probably because they are naturally more permeable and uptake higher concentrations of the CPA. Such stage-dependent permeability in embryos was reported in medaka [21], turbot [22,23], winter flounder [24], and in our previous studies with whiting [25]. Increasing diameter of chorion canals during embryo development could have accounted for enhanced CPA permeation [26].

In this study, the DMSO content of the embryos increased with increasing concentration of DMSO in the impregnation medium. For example, the DMSO content of embryos (somites-stage) electroporated at 300 V in 10%, 20%, or 30% DMSO solutions were 10, 30, and 78 mM, respectively. Concentration-dependent permeation was also reported in nonelectroporated embryos by Magnus et al. [27], Suzuki et al. [16], Cabrita et al. [22], and Rahman et al. [25], for carp, medaka, turbot, and whiting embryos, respectively. Embryos treated with higher voltages (400 or 500 V) had even greater DMSO uptake but poorer viability, as previously mentioned. Conversely, pre-exposure of embryos to DMSO solution before electroporation not only improve DMSO uptake, but also minimized embryo mortality compared with other strategies. Thus, somites-stage embryos pre-exposed to 10% DMSO for 20 minutes followed by electroporation in 30% DMSO had a 41% increase in CPA uptake compared with those without pre-exposure and approximately a 152% increase compared with those treated with only 10% DMSO for 20 minutes [25]. Pre-exposure with DMSO might equilibrate the water and CPA movement between intra- and extraembryonic fluid [28]. Nevertheless, even embryos treated under the optimum conditions established in this study did not tolerate rapid cooling to cryogenic temperatures and only a few embryos had apparently normal morphology after thawing. Therefore, we inferred that the internal DMSO concentrations attained with our treatments were not sufficient to prevent damaging ice formation during cooling and thawing. Thus, further studies should attempt to combine the advantages of electroporation with those of other methods previously shown to increase CPA uptake, e.g., impregnation in the presence of inorganic salts ($CaCl_2$ or $MgCl_2$) and pre-treatment of embryos with nonpermeating agents, e.g., trehalose [25].

4.1. Conclusions

The best results in terms of embryo viability and DMSO uptake were obtained when whiting embryos were exposed to electroporation at 300 V in 30% DMSO. Pre-exposure of embryos to 10% DMSO solutions before electroporation further enhanced cryoprotectant permeation into the embryos without compromising viability. Overall, we inferred that electroporation might be effective to promote CPA uptake by fish embryos, but it might require a combination with other treatments to attain sufficient internal CPA concentrations to prevent cryoinjuries during cryopreservation.

Acknowledgments

This study was supported by a FY2011 JSPS postdoctoral fellowship for foreign researchers (P 11092) and by a scholarship to Sk. Mustafizur Rahman, from the Ministry of Education, Culture, Sports, Science and Technology (Monbukagakusho) of Japan. The authors thank Y. Takeuchi and the staff of the Field Science Center, Tateyama Station (Banda), Tokyo University of Marine Science and Technology, for kindly supplying Japanese whiting broodstock.

References

[1] Robles V, Cabrita E, Real M, Ivarez R, Herráez MP. Vitrification of turbot embryos: preliminary assays. Cryobiology 2003;47:30–9.
[2] Cabrita E, Robles V, Wallace JC, Sarasquete MC, Herráez MP. Preliminary studies on the cryopreservation of gilthead seabream (*Sparus aurata*) embryos. Aquaculture 2006;251:245–55.
[3] Edashige K, Valdez Jr DM, Hara T, Saida N, Seki S, Kasai M. Japanese flounder (*Paralichthys olivaceus*) embryos are difficult to cryopreserve by vitrification. Cryobiology 2006;53:96–106.
[4] Ding FH, Xiao ZZ, Li J. Preliminary studies on the vitrification of red sea bream (*Pagrus major*) embryos. Theriogenology 2007;68:702–8.
[5] Zhang T, Rawson DM. Studies on chilling sensitivity of zebrafish (*Brachydanio rerio*) embryo. Cryobiology 1995;32:239–46.
[6] Hagedorn M, Hsu E, Kleinhans FW, Wildt DE. New approaches for studying the permeability of fish embryos: toward successful cryopreservation. Cryobiology 1997;34:335–47.
[7] Leung LKP. Principles of biological cryopreservation. In: Jamieson BGM, editor. Fish evolution and systematics: evidence from spermatozoa. Cambridge: Cambridge University Press; 1991. p. 231–44.
[8] Leung LKP, Jamieson BGM. Live preservation of fish gametes. In: Jamieson BGM, editor. Fish evolution and systematics: evidence from spermatozoa. Cambridge: Cambridge University Press; 1991. p. 245–69.
[9] Hagedorn M, Peterson A, Mazur P, Kleinhans FW. High ice nucleation temperature of zebrafish embryos: slow-freezing is not an option. Cryobiology 2004;49:181–9.

[10] Eide FF, Eisenberg SR, Sanders TA. Electroporation-mediated gene transfer in free-swimming embryonic *Xenopus laevis*. FEBS Lett 2000;486:29–32.
[11] Shirakashi R, Sukhorukov VL, Tanasawa I, Zimmermann U. Measurement of the permeability and resealing time constant of the electroporated mammalian cell membranes. Int J Heat Mass Transfer 2004;47:4517–24.
[12] Zimmermann U, Pilwat G, Riemann F. Reversible dielectric breakdown of cell membranes by electrostatic fields. Z Naturforsch 1974; 29c:304–5.
[13] Shirakashi R, Köstner CM, Muller KJ. Intracellular delivery of trehalose into mammalian cells by electropermeabilization. Membrane Biol 2002;189:45–54.
[14] Rahman SM, Majhi SK, Suzuki T, Strüssmann CA, Takai R. Basic studies on the cryopreservation of Japanese whiting (*Sillago japonica*) embryos. Cryobiology 2007;55:347.
[15] Rahman SM, Majhi SK, Suzuki T, Matsukawa S, Strüssmann CA, Takai R. Suitability of cryoprotectants and impregnation protocols for embryos of Japanese whiting *Sillago japonica*. Cryobiology 2008; 57:170–4.
[16] Suzuki T, Komada H, Takai R, Arii K, Kozima TT. Relation between toxicity of cryoprotectant Me_2SO and its concentration in several fish embryos. Fish Sci 1995;61:193–7.
[17] Oozeki Y, Hirano R. Effects of temperature changes on the development of eggs of the Japanese whiting *Sillago japonica* Temminck et Schlegel. Bull Jap Soc Sci Fish 1985;51:557–72.
[18] Shigekawa K, Dower WJ. Electroporation of eukaryotes and prokaryotes: a general approach to the introduction of macromolecules into cells. Biotechniques 1988;6:742–51.
[19] O'Hare MJ, Ormerod MG, Imrie PR, Peacock JH, Asche W. Electropermeabilization and electrosensitivity of different types of mammalian cells. In: Neuman E, Sowers AE, Jordan CA, editors. Electroporation and electrofusion in cell biology. New York: Plenum Press; 1989. p. 319–41.
[20] Sin FYT, Bartley AL, Walker SP, Sin IL, Synmonds JE, Hawke L, et al. Gene transfer in chinook salmon (*Oncorhynchus tshawytscha*) by electroporating sperm in the presence of pRSV-lacZ DNA. Aquaculture 1993;117:59–69.
[21] Routray P, Suzuki T, Strüssmann CA, Takai R. Factors affecting the uptake of DMSO by the eggs and embryos of medaka, *Oryzias latipes*. Theriogenology 2002;58:1483–96.
[22] Cabrita E, Robles V, Chereguini O, de Paz P, Anel L, Herráez MP. Dimethyl sulfoxide influx in turbot embryos exposed to a vitrification protocol. Theriogenology 2003;60:463–73.
[23] Cabrita E, Chereguini O, Luna MJ, de Paz P, Herráez MP. Effect of different treatments on the chorion permeability to DMSO of turbot embryos (*Scophthalmus maximus*). Aquaculture 2003;221:593–604.
[24] Robles V, Cabrita E, Fletcher GL, Shears MA, King MJ, Herráez MP. Vitrification assays with embryos from a cold tolerant sub-arctic fish species. Theriogenology 2005;64:1633–46.
[25] Rahman SM, Strüssmann CA, Majhi SK, Suzuki T, Watanabe M. Efficiency of osmotic and chemical treatments to improve the permeation of the cryoprotectant dimethyl sulfoxide to Japanese whiting (*Sillago japonica*) embryos. Theriogenology 2011;75:248–55.
[26] Depeche J, Billard R. Embryology in fish, a review. Paris: Société Française d'Ichtyologie; 1994.
[27] Magnus Y, Ar A, Lubzens E. Permeability and toxicity of ^3H DMSO to developing ornamental carp eggs. In: Goetz FW, Thomas P, editors. Proceedings of the Fifth International Symposium on the Reproductive Physiology of Fish. Austin: FishSymp95; 1995. p. 128.
[28] Wang RY, Guan M, Rawson D, Zhang T. Ultrasound enhanced methanol penetration of zebrafish (*Danio rerio*) embryos measured by permittivity changes using impedance spectroscopy. Eur Biophys J 2008;37:1039–44.