CRYOPRESERVATION OF EUROPEAN EEL *(ANGUILLA ANGUILLA)* SPERM USING DIFFERENT EXTENDERS AND CRYOPROTECTANTS

SHORT COMMUNICATION

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Experiments were carried out on the sperm cryopreservation of artificially induced eels. The effects of several extenders and two cryoprotectants on the motility of spermatozoa were investigated. The highest post-thaw motility was observed with the combination of Tanaka's extender and DMSO as cryoprotectant. Further dilution after thawing resulted in complete loss of motility in samples frozen in presence of DMSO while sperm frozen with methanol as cryoprotectant retained its motility after further dilution.

Keywords: Eel – sperm – cryopreservation – methanol – DMSO

Induced eel spawning is still problematic as up to this date successful rearing to glass eel stage has not been reported. Hormonally induced sexual maturation of the eel is not synchronous in the two sexes. Sperm cryopreservation can resolve the problem if synchronization of sexual maturation. Ohta and Izawa [2] reported on the successful short-term preservation of Japanese eel *(Anguilla japonica)* sperm. A method of long-term cryopreservation of Japanese eel sperm was developed by Tanaka et al. [3], however, there is no standard method of cryopreservation of European eel *(Anguilla anguilla)* sperm. The objective of this study was to develop a reproducible method of cryopreservation of European eel sperm.

Six males in two groups were used for cryopreservation experiments (in 60 l tanks with internal filtering, natural illumination, water temperature: 24–26 °C, salt concentration: 0.05%). The fish were anaesthetized using pheoxy-ethanol (40 ml dissolved in 100 l of water) then they were injected 9 times with a uniform dose of human chorion gonadotropine (250 IU per individual per week dissolved in 0.9 NaCl solution). Sperm was collected for cryopreservation after the 8. and 9. treatments. Fish were anaesthetized with phenoxy-ethanol, then the area of the urogenital opening was wiped dry with a cloth. Sperm was collected with an automatic pipette and distributed into plastic test tubes. Sperm motility was estimated following activation with artificial saltwater (3.5% NaCl solution) on a glass slide under ×200 magnification.

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The following extenders were prepared: 1. 350 mM glucose, 30 mM Tris (pH 8.0); modified Kurokura's extender [1]: 350 mg NaCl, 1000 mg KCl, 22 mg $CaCl_2$, 8 mg $MgCl_2$ and 20 mg $NaHCO_3$ (for 100 ml); Tanaka's extender [3]: 137 mM NaCl, 76.2 mM $NaHCO_3$. Ten per cent dimethyl sulfoxide (DMSO) or ten per cent methanol (in v/v final concentration) was used as cryoprotectant. Sperm was diluted 1 : 9 with the cryodiluent containing the extender and the cryoprotectant. Diluted sperm was loaded into 250 µl straws. One ml (four straws) were frozen of each treatment in the vapor of liquid nitrogen in a styrofoam box. Straws were placed onto a styrofoam frame (height: 4 cm) floating on the surface of liquid nitrogen and after 3 minutes they were plunged directly into liquid nitrogen. Straws were thawed in a 40 °C water bath for 5 seconds 3–4 hours after freezing. A sample of each treatment was diluted in a 1 : 9 ratio with the extender in which it was frozen. Thus after thawing we had two samples of each treatment: one undiluted and one diluted. Motility of these samples was analyzed as described above.

Post-thaw motility data were subjected to two-way analysis of variance (ANOVA) to investigate the main effects of cryoprotectants and extenders. The statistical analysis was carried out using GraphPad Prism 4.0 statistical software.

Motility of fresh sperm samples used for cryopreservation (0.8–1.3 ml per animal) was 65 ± 5%. The highest post-thaw motility (47 ± 15%) was observed with the combination of Tanaka's extender and DMSO as cryoprotectant (Table 1). Similar motility results (40 ± 10%) were found with the combination of Tanaka's extender and methanol. All other combinations resulted in poorer post-thaw motility rates. We found that cryoprotectants did not have a significant effect on the post-thaw motility of undiluted eel sperm but they had a significant effect ($P < 0.0001$) on the motility of sperm that was diluted following thawing. Extenders had a significant effect on the post-thaw motility of both undiluted ($P < 0.0001$) and diluted ($P = 0.0013$) sperm.

In the present study we found that Tanaka's extender specifically developed for Japanese eel sperm resulted in the highest post-thaw motility rates of European eel

Table 1

Post-thaw of European eel sperm frozen in presence of three extenders (Tanaka's extender, modified Kurokura's extender and glucose extender) and two cryoprotectants (dimethyl sulfoxide – DMSO and methanol)

		Tanaka	Modified Kurokura	Glucose
DMSO	Undiluted	47 ± 15^{a1}	8 ± 3^{b1}	3 ± 6^{b1}
	Diluted	0 ± 1^{a2}	2 ± 3^{a2}	0 ± 0^{a2}
Methanol	Undiluted	40 ± 10^{a1}	12 ± 8^{b1}	3 ± 3^{b1}
	Diluted	40 ± 10^{a1}	18 ± 13^{a1}	2 ± 3^{b12}

The sperm was either analysed without dilution or diluted 1 : 9 with the corresponding extender. Values sharing a superscript letter within a row were not significantly different ($P > 0.05$). Values sharing a superscript number within a column were not significantly different ($P > 0.05$).

sperm. The glucose and modified Kurokura's extender that were successfully used on common carp sperm gave lower motility values. While cryoprotectants did not have a significant effect on the post-thaw motility of undiluted eel sperm, dilution of sperm following thawing significantly reduced the motility of sperm frozen with DMSO as cryoprotectant. This reduction was not observed when methanol was used as cryoprotectant. A possible explanation of this observation is the change in osmolality of sperm following dilution. While methanol does not affect the osmolality of extenders, the addition of 10% DMSO to modified Hanks' Balanced Salt Solution (HBSS) increased its osmolality from 270 mOsmol/kg to 1568 mOsmol/kg [4]. Eel spermatozoa seem to tolerate this increase in osmolality as thawed and undiluted eel sperm was motile following activation with artificial saltwater. However further dilution with a solution that has a significantly lower osmolality seems to cause an osmotic shock to the cells that inhibits their motility. Dilution of sperm following thawing reduces the toxicity of cryoprotectants thus it can be important for short-term (1–10 days) storage of thawed sperm. The use of methanol as cryoprotectant seems to be more suitable for this purprose.

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