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Japanese eel (*Anguilla japonica* Temminck & Schlegel, 1846) propagation by using cryopreserved sperm samples

Short communication

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Running title: Japanese eel propagation by using cryopreserved sperm

Summary

Our aim of this study was to test the artificial seminal plasma (ASP) as extender and methanol as cryoprotectant for cryopreservation and to collect information on the fertilizing capacity of cryopreserved sperm samples. Eggs from Japanese eel females were controlled fertilized with native sperm diluted with ASP and cryopreserved sperm (ASP as extender (in 1:100 ratio) and 10% methanol in v/v final concentration). There were no statistical differences ($p<0.05$) among the measured parameters (hatching, survival rates after 10 days post-hatch, malformation) between two groups so this cryopreservation method can be effectively used for artificial propagation of Japanese eel as well.

Introduction

The induction of sexual maturation in captivity is a long process promoted by weekly hormone injections. The synchronization of ovulation and spermiation is not easy; thus, the cryopreservation of sperm would render the synchronization of spermiation and ovulation unnecessary (Müller et al., 2012). Tanaka et al. (2002) were the first to publish and apply a practical way of cryopreservation of Japanese eel sperm (*Anguilla japonica*). Cryopreserved sperm containing 76.2 mM $NaHCO_3$, 137mM NaCl, 1.4% Soya lecithin and 10% dimethyl sulphoxide (DMSO) was used as cryoprotectant. Müller et al. (2012) applied modified Tanaka's extender containing 350 mM glucose, 30 mM Tris (pH 8.0) and methanol cryoprotectant for cryopreservation of European eel sperm samples and it was used for hybridization between Japanese eel and European eel resulting hatched hybrid larvae. Asturiano et al (2016) managed to get European eel larvae by using 50 mM NaCl, 100 mM $NaHCO_3$, 2 mM MgCl2, 1mM CaCl2, 30 mM KCl, pH 6.5, mOsm/kg ~330 as media and Me2SO 10% (v/v) and 25% foetal bovine serum (w/v).

While there are great differences between the weights of the stripped eggs (300-500 g) and sperm volume (1-3 g) of Japanese eel therefore, Ohta et al. (1997) suggested and used artificial seminal plasma (ASP) to dilute the milt, which correspond - in ionic constituents - to the seminal plasma and pH of the milt of artificially matured Japanese eel. Our aim of this study was to test the ASP as extender and methanol as cryoprotectant for cryopreservation and to collect more information on the fertilizing capacity of cryopreserved sperm samples as well as malformation rate.

Material and methods

The maturation and cryopreservation of Japanese eel were performed in the Tokyo University of Agriculture, Abashiri, Japan. The method of full sexual maturation of farmed Japanese eel males was described by Ohta et al. (1997; maturation temperature 20 °C, tank size 2500L, two years old males, n=4, BW= 250-300 g). 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. Sperm samples showing motility higher than 70% were chosen for cryopreservation. A freezing diluent was prepared in a test tube containing ASP (149.3 mM NaCl + 15.2 mM KCl + 1.3 mM CaCl2 + 1.6 mM MgCl2 + 20 mM NaHCO3 buffered with 20 mM TAPS-NaOH at pH 8.1, (Ohta et al. 1997). The milt was mixed with ASP in 1:100 ratio (average cell contcentration 61692 / mL). Ten per cent methanol in v/v final concentration was used as cryoprotectant. Sperm was loaded into 0.5 mL straws immediately after dilution in room temperature (20 °C). Samples were frozen in the vapor of liquid nitrogen in an insulated polystyrene box. Liquid nitrogen was poured into the box and a polystyrene frame was placed onto the surface of liquid nitrogen. The height of frame was 3 cm. Straws were placed onto the frame for 3 min (-160 °C), and then

they were plunged directly into liquid nitrogen (-196 °C). After freezing samples had been kept into storage Dewar bottles and kept for 1-3 days before it was used. The protocol by Ohta et al. (1997) with slight modification by Abe et al. (2010) was applied for induction of sexual maturation and ovulation of Japanese eel. Eggs, which originated from four females (initial body weight was 578.0 ±129.4 g), were collected into dry plastic bowls by gentle abdominal pressure. Eggs were distributed into 5 cm Ø 50 ml beaker in batches (1 g) of approximately 1700-1800 eggs. Straws were thawed for 13 seconds in a water bath at 40 ºC. Eggs were fertilized with thawed sperm samples and same amount native Japanese eel sperm diluted with ASP samples in 1:100 ratio as control. Sperm was added to the eggs and then 10 mL of artificial seawater (NAPCO instant or ocean Premium) was used to activate the gametes. According to Unuma et al. (2004) fertilization method was used. After fertilization egg samples from four females were incubated in 100 mL buckets for archives of deformation types and 2×48-well microplates (1 egg/well) for reveal reproduction parameters (hatching rate, survival rate, malformation rate) in a thermostat (25 °C).

The following parameters were recorded according to Kurokawa et al. (2008) and Okamoto et al. (2009).

Hatching rate = 100 × number of hatched larvae / the number of incubated eggs

Spinal malformations in tail fin and pericardium oedemas were assessed at hatching by observation with a dissecting microscope and the following rates were calculated:

Malformation rate 1. = 100× number of hatched malformed larvae / number of total eggs

Malformation rate 2. = 100 × number of malformed larvae/number of surviving larvae

Statistical analyses were carried out with SPSS for Windows. Independent Samples Test was used to test the main effects of the treatments using alpha = 0.05 for significance.

Results

The mass larvae hatching were 28-30 hours after fertilization. There were no statistical differences ($p>0.05$) among the measured parameters such as hatch and survival after 10 days post-hatch rates due to the large individual fluctuations (Table 1.). The malformation rate of larvae compared to the hatching rate was higher in cryopreserved group but statistical differences were not observed between the groups.

Discussion

ASP as extender and methanol as cryoprotectant yielded 6.2 – 32.6% hatch in this experiment comparing to fresh sperm (4.2 – 29.2%), which results were similar to Tanaka et al (2002), who used “Tanaka solution” and DMSO as cryoprotectant: the hatchability of eggs fertilized with cryopreserved sperm was generally low (2.4 – 22.5%, n= 3 females) compared to fresh sperm (11.6 – 32.5%). This result was better than previous one (Müller et al 2012), where only some hybrid larvae hatched (hatching rate <1%). Although the rearing water temperature and salinity effects on rate of larvae malformation in Japanese eel have been observed previously too (Kurokawa et al. 2008; Okamoto et al. 2009) but embryo incubation and larvae rearing of our study was kept in the same condition in this experiments. The malformation was higher in larvae originating from cryopreserved fertilisation but there was no significant differences ($p>0.05$). The advantage of the applied method is

ASP generally use for artificial propagation of Japanese eel so there is not important to make two different types of solution and dilution.

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Table 1. Summarised base data of the reproduction parameters among the investigated groups (Malformation rate 1. = number of hatched malformed larvae / number of total eggs × 100; Malformation rate 2. = number of hatched malformed larvae / number hatched larvae × 100. From fertilisation to 10 days old larvae were reared in 25 °C)

Fertilisation with sperm from		Hatch rate (%)	Survival rate after 10 days hatch (%)	Malformation rate 1.	Malformation rate 2.
		n=4			
ASP	mean ± SD	19.8 ± 11.2	14.3 ± 10.7	5.4 ± 3.3	32.5 ± 28
	min – max	4.2 – 29.2	0.7 – 25	2.1 – 10.4	16 – 49.9
CRYO	mean ± SD	16.9 ± 11.2	8.6 ± 4.9	7.7 ± 6.2	41 ± 18.4
	min – max	6.3 – 32.3	2.5 – 12.6	1.0 – 15.6	19.9 – 62.5