

Preliminary Studies on Cryopreservation of Common Tench (*Tinca tinca*) Embryos (Work in Progress)

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Abstract: Vitrification could provide a promising tool for the cryopreservation of fish embryos. However, in order to achieve cryopreservation using vitrification, chilling sensitivity and cryoprotectants toxicity were determined using tench embryos at four developmental stages (eleven, seventeen, twenty three and twenty nine hour). Embryos treated with alcalase (2 mL/998 mL, 2 min at 22 °C) were exposed to chilling with/without warming. Other embryos were exposed to methanol and glycerol at the concentration of 10 and 20% for periods of 20 min. At last embryos were incubated at special incubator cages where hatching rates were counted. Regarding chilling sensitivity, the hatching rates of embryos decreased significantly ($p < 0.001$) after exposure to 0°C at all developmental stage except 29-h stage compared with the controls. The same results were reported after exposure to chilling followed by warming. The embryo stage most sensitive to chilling was 11-h stage. The 29-h stage exhibited the least sensitivity to low temperature while 17-h and 23-h stages were intermediate in their sensitivity to chilling. The toxicity of methanol increased significantly ($p < 0.001$) with developmental stage for 11-h, 17-h and 23-h stages. The largest change was a reduction in the toxicity of methanol in 29-h embryos. The highest hatching rates of tench embryos were obtained with 29-h embryos using various concentrations of methanol. The survival trends of different stages of tench embryos exposed to glycerol concentrations were approximately similar to those embryos exposed to methanol concentrations except for 11-h embryos which showed no hatching with both glycerol concentrations. Concerning the viability of embryos after vitrification, unfortunately, we could not obtain viable embryos in any of conditions examined. In conclusion, it was quite difficult to vitrify the tench embryos during this study using various vitrifying solutions and the method reported by Chen and Tian and further studies using other developmental stages, basic, washing and vitrifying solutions are needed to achieve successful cryopreservation.

Key words: Tench, cryoprotectant, hatching, viability, vitrification

INTRODUCTION

Cryopreservation of fish embryos could play a major role in seed production, genetic management of broodstock and conservation of aquatic resources. Although a few studies have claimed successful cryopreservation of fish embryos (Wang *et al.*, 1987; Zhang *et al.*, 1989; Zhang *et al.*, 1997) there are no reproducible cryopreservation protocols (Rana, 1995).

Conventional cryopreservation of fish embryos has been unsuccessful, which may be due to their large size (1-7 mm diameter), multi-compartmental structure, low water permeability that lead to intracellular ice formation, sensitivity to chilling injury and high yolk content in the

egg (Gwo 2000). To overcome these obstacles, vitrification, the rapid cooling of liquid medium in the absence of ice crystal formation using a highly concentrated solution of cryoprotectant and rapid cooling of the embryo suspension, was used recently by Chen and Tian (2005) to achieve successful cryopreservation of Japanese flounder (*Paralichthys olivaceus*) embryos.

Before their report, there had been only one report on common carp (*Cyprinus carpio*) embryos surviving cryopreservation using a programmed slow freezing method in liquid nitrogen (Zhang *et al.*, 1989) but results have not been reproducible.

Stage-dependent chilling sensitivity has been reported for many species of fish embryos, including

rainbow trout (*Oncorhynchus mykiss*) (Haga 1982), carp (*Cyprinus carpio*) (Dinnyes *et al.*, 1998), fathead minnows (*Pimephales promelas*) (Cloud *et al.*, 1988), goldfish (*Carassius auratus*) (Liu *et al.*, 1993) and zebrafish (*Danio rerio*) (Zhang and Rawson 1995). The early developmental stages of the zebrafish were most sensitive to chilling injury (Zhang and Rawson 1995). Cabrita *et al.* (2003) found in turbot that late-stage embryos appeared to be more tolerant to cryoprotectant than earlier stages while Chen and Tian (2005) reported in flounder that late-stage embryos were less tolerant to vitrifying solutions than the other developmental stages. The latter recorded also that five-step equilibration of flounder embryo in vitrifying solution containing propylene glycol and methanol resulted in high survival rates.

Zhang *et al.* (2005) reported that the sequence of cryoprotectants toxicity to flounder embryos was: Propylene glycol < methanol < dimethyl sulfoxide < glycerol. A similar sequence of toxicity in zebrafish (Zhang *et al.*, 1993) and in sea perch (Tian *et al.*, 2003) was recorded. Dinnyes *et al.* (1998) found that the toxicity to carp embryos increased in the order of methanol < dimethyl sulfoxide < glycerol in 4-h and 8-h embryo stages while it increased in the order of methanol < glycerol < dimethyl sulfoxide in 32-h embryo stage.

Tench, *Tinca tinca*, is a Cyprinid that is wide-spread in Europe and adjacent regions.

Cryopreservation of tench embryos would be beneficial for conservation of germplasm and genetic improvement of freshwater fish. In addition, tench eggs are relatively small, with a diameter of 0.8-1.2 mm (Yilmaz, 2002).

To our knowledge, no available literature on chilling sensitivity, toxicity of cryoprotectants and cryopreservation of tench embryos has been reported. So the present study aimed to test chilling sensitivity, the toxicity of methanol and glycerol at the concentration of 10 and 20% and the survival after vitrification of tench embryos.

MATERIALS AND METHODS

All experiments were carried out in the experimental hatchery of the University of South Bohemia, Research Institute of Fish Culture and Hydrobiology, Department of Fish Genetics and Breeding at Vodnany, Czech Republic.

Chemicals: All chemicals used were of high purity and were purchased from Sigma-Aldrich Co. (Deisenhofen, Germany). Alcalase enzyme, Bacillus licheniformis cat. no. 12674120 were purchased from Calbiochem, CA, USA.

Tench culture and embryos collection under hatchery condition: Five to seven year-old broodstock (400-700 g

body weight, Hungarian strain) were caught from breeding ponds in April, brought into the hatchery, kept in two natural ponds (0.01 ha), fed with pelleted feed for carp and monitored for sexual development by means of abdominal compression. Fish were separated by sex and those fish exhibiting gonadal maturity were introduced to spawning induction. Spawnable broodstock were selected in June and held in two indoor flow-through tanks (4000 l), supplied with irrigation water (temperature 18-22 °C and O₂ 6-7 mg L⁻¹) at a flow rate of 0.2 L/s.

Spermiation was induced by one injection of 1•0 mg kg⁻¹ b.w. Carp Pituitary Extract (CPE) dissolved in physiological solution. Ovulation was also induced by intramuscular injection of 5 µg kg⁻¹ b.w. of GnRH analogue (D-Ala⁶, GnRH ProNH₂Et, Kobarelin). Prior to each injection and gamete collection, the males and females were anaesthetized in a solution of 2-phenoxyethanol (1:1000).

Thirty hours post-injection, ovulated eggs stripped from 9 females were pooled and stored approximately 1 h at a temperature of 17-18 °C until fertilization. Twenty four hours after CPE treatment, sperm was collected individually from the genital papilla from each of 10 males into 5 mL syringes containing 2 mL of the immobilization solutions (IS; Kurokura 180 = 180 mM NaCl, 2.68 mM KCl, 1.36 mM CaCl₂ and 2.38 mM NaHCO₃) and stored for 4-8 h at 4-6°C (Rodina *et al.*, 2004). Eggs and Sperm of all females and males were pooled in the same proportions by volume as that obtained by stripping.

The mixture of 500 g of eggs and 5 mL sperm with IS (rate 0.5:1) was directly activated with 500 mL of clean hatchery water at an optimum temperature 22 °C. After 2.5 min, the excess solution was poured out. During that time the eggs were hydrated and swelled rapidly and egg sticking was prevented during that period by constant mixing. For the elimination of egg stickiness alcalase enzyme is used 3 min. after fertilization. Optimum rates between eggs and diluted enzymes (2 mL of alcalase enzyme diluted in 998 mL of hatchery water) is 1:1 (g eggs: mL of diluted enzymes) with stirring for 2 min. After 2 min of exposure in enzyme solution, the eggs are rapidly rinsed with hatchery water and transferred to Weis jars and incubated at 22 °C.

Before doing the experiments, three batches of approximately 0.2 g (around 300 eggs) of pooled unfertilized eggs were weighed to the nearest 0.0001 g and fixed in 4 % formaldehyde for later counting and determination of mean egg weight. Based on this, the number of eggs in a sample was expressed as the weight of the sample (g) multiplied by 1600.

Experimental design: A total of 28,800 morphologically normal tench embryos at different developmental stages (11, 17, 23 and 29 h) were used for the experiments of chilling sensitivity and toxicity of cryoprotectants. Experiments were performed in 3 replicates and each

treatment group consisted a total of 3600 embryos. Moreover, 5,500 embryos at 23 and 29 h stages were used for the experiments of vitrification.

Basic solutions: Hatchery water served as basis for the Vitrifying Solutions (VS) instead of artificial seawater used by Tian and Chen (2005) as tench fish is freshwater fish.

Preparation of washing solution: Washing solution was prepared by dissolving 0.125 M sucrose in hatchery water.

Experiments on the chilling sensitivity: Chilling group consisted of 300 embryos (from each stage and at each replicate) in 10 mL of hatchery water in 15 mL plastic test-tube were placed into ice water-bath at 0°C for 20 min. Other treatment group (chilling-warming) was composed of 300 embryos in 10 mL of hatchery water in 15 mL plastic test tubes were placed into water-bath at 0°C for 10 min then were placed in water-bath at 22°C for another 10 min. After each treatment, embryos were washed twice with washing solution and were transferred to small special incubator cages of 200 cm², each supplied with recirculated water at 22°C and 9 mg oxygen⁻¹ and left till hatching (usually up to 4 days after gametes activation). The temperature of each solution after equilibration ($\pm 0.5^\circ\text{C}$) was confirmed by a small portable digital thermometer.

The percentage of Hatching rate (H_i) was calculated for each cage from the number of Hatched larvae (H_i) divided with the total number of eggs placed in the cage (E_i) as follows:

$$H_i = (H_i/E_i).100$$

Control embryos were manipulated in room-temperature water in a similar manner then transported to the incubator cages.

Experiments on the toxicity of cryoprotectants: The sensitivity of embryos to the toxicity of individual cryoprotectants was examined by a method similar to that reported by Chen and Tian (2005). Embryos at four different stages were exposed to cryoprotectant solutions (hatchery water containing 10% (vol vol⁻¹) methanol, 20% (vol vol⁻¹) methanol, 10% (vol vol⁻¹) glycerol, or 20% (vol vol⁻¹) glycerol) at 22°C for 20 min. The embryos were then transferred to washing solution at 22°C, kept for 10 min and were then transferred to the incubation cages to calculate the hatching %.

Vitrification of embryos in different vitrifying solutions: Eight Vitrifying Solutions (VS₁-VS₈) were prepared using methanol alone, Glycerol alone or by combining both in

different proportions. The vitrification solutions, VS₉-VS₁₁, were prepared according to modified Chen and Tian's method (2005). The composition of these vitrification solutions are shown in Table 1.

Based on our results on cryoprotectants toxicity, 150 embryos at each stage of 23-h and 29-h stages were used to test the vitrifying solutions (VS₁-VS₁₁). Embryos were stepwise equilibrated following Chen and Tian's method (2005) in 5 step method 25, 33, 50, 67 and 100 % concentration level of vitrifying solution for 8 min in each step at 22°C and washed in washing solution for 10 min. Following osmotic equilibration in each vitrifying solution, 15-20 embryos were loaded into 0.5 mL plastic straw (IMV, France) using micropipette 1 mL. The straws were heat-sealed then plunged directly into Liquid Nitrogen (LN₂) (-196 °C) in a 15 L liquid nitrogen streobox for 5-20 min. Successful versus unsuccessful vitrification was assessed by visual inspection according to Chen and Tian's method (2005). Presence or absence of intra cellular ice crystals was monitored by opacity resulted from whitening of the solution or transparency of embryos.

In some experiments, 100 embryos at each stage of 23-h and 29-h stages were treated stepwise with VS₁-VS₁₁ solutions and directly plunged into propylene beakers filled with LN₂ and kept for 5-20 min.

Thawing and incubation of embryos: Straws containing frozen embryos were quickly removed from LN₂ and immersed in a water bath at 40°C for rapid thawing (about 9 s). After thawing, the 2 ends of the straw were cut and embryos were placed in 2 mL of washing solution for 10 min. About 8 mL of hatchery water at 22°C was slowly added to dilute the thawed embryos, and after 10 min the thawed embryos were transferred to hatchery water in the incubator cages until hatching or death. For these embryos plunged directly in beakers with liquid nitrogen, they were thawed by direct immersion on incubator cages to assess hatching.

Statistical analysis: Means of the data acquired were evaluated from 3 replicates. Data is presented as mean \pm Standard Error of the Mean (SEM). Statistical significance was assessed using multiple Analysis of Variance (ANOVA, Statgraphics version 5), followed by multiple comparison LSD range test. Probability values < 0.05 were considered significant. The statistical analysis was computed using SPSS software.

RESULTS

Sensitivity of embryos to chilling with/without warming: Figure 1 presents the results on chilling sensitivity with/without warming at various developmental stages. The hatching rates of embryos decreased significantly

Table 1: Composition and properties of vitrifying solutions for tench embryos

	VS ₁	VS ₂	VS ₃	VS ₄	VS ₅	VS ₆	VS ₇	VS ₈	VS ₉	VS ₁₀	VS ₁₁
HW (%)	90	80	90	80	80	70	70	60	67	60	55
MeOH(%)	10	20	0	0	10	10	20	20	13	16	18
Gly (%)	0	0	10	20	10	20	10	20	0	0	0
PG (%)	0	0	0	0	0	0	0	0	20	24	27

The units of composition are % by volume, HW = Hatchery Water, MeOH = Methanol, Gly = Glycerol, PG = Propylene Glycol, VS = Vitrifying Solutions,

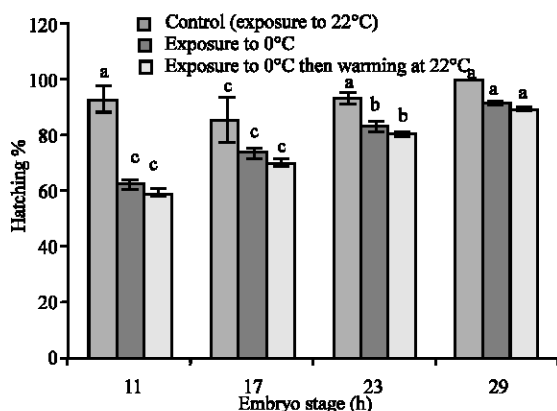


Fig. 1: Hatching rates of tench embryos following exposure to 0°C with/without warming. Groups with the same superscript do not differ significantly (p < 0.05)

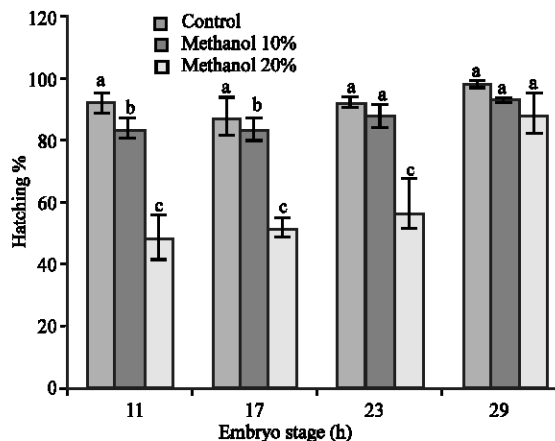


Fig. 2: Hatching rates of tench embryos following exposure to 10 and 20% methanol at 22°C. Groups with the same superscript do not differ significantly (p < 0.05)

(p < 0.001) after exposure to 0°C at all developmental stage except 29 h stage compared with the controls. The same results were reported after exposure to chilling followed by warming. The embryo stage most sensitive to chilling was 11 h stage. The 29 h stage exhibited the least sensitivity to low temperature while 17 and 23 h stages were intermediate in their sensitivity to chilling.

Sensitivity of embryos to the toxicity of cryoprotectants:

Data analysis showed that developmental stage and cryoprotectants had a significant effect on survival.

Data on the toxicity of methanol at 22°C are presented in Fig. 2. The toxicity of methanol increased significantly (p < 0.001) with developmental stage for 11, 17 and 23 h stages. The largest change was a reduction in the toxicity of methanol in 29-h embryos. Figure 2 also shows that when 10% methanol was used, it reduced the hatching rates of 11, 17 and 23 h embryos significantly (p < 0.05) while it results in a non significant decrease of hatching rate of 29 h embryos compared with the controls. On using 20% methanol, it decreased the hatching rates of 11, 17 and 23 h embryos significantly (p < 0.001) while it decreased the hatching rate of 29-h non significantly compared with the controls. The highest hatching rates of tench embryos were obtained with 29 h embryos using various concentrations of methanol.

The effects of exposure to glycerol at 22°C are showed in Fig. 3. The tench embryo stage most sensitive to glycerol toxicity was 11 h which showed no hatching with both 10 and 20 % glycerol. The 29 h stage exhibited high tolerance to glycerol toxicity while 17 and 23 h stages were intermediate in their tolerance to toxicity. The survival trends of different stages of tench embryos exposed to glycerol concentrations were approximately similar to those embryos exposed to methanol concentrations except for 11 h embryos which showed no hatching with both glycerol concentrations.

Survival of embryos after vitrification: Table 1 shows the composition and properties of vitrifying solutions used during this study to cryopreserve tench embryos.

As preliminary experiments, vitrifications solutions were cooled and warmed in straws without embryos to check the ice formation (Table 2). When the solutions in straws were cooled by being plunged into LN₂, VS₁-VS₈ and VS₁₀ became opaque partially or entirely but VS₉ and VS₁₁ remained transparent during cooling, suggesting that ice crystals were not formed in VS₉-VS₁₁ during cooling. During warming at 40°C, VS₁-VS₈, VS₁₀ and VS₁₁ became opaque partially or entirely but VS₉ remained transparent, suggesting that VS₉ remained uncrystallized during warming.

Table 2: Formation of ice crystals in vitrification solution during cooling and warming in straws

Container	Vitrification solutions	Cooling	Warming	Container	Vitrification solutions	Cooling	Warming
Straws	VS ₁	+	+	Straws	VS ₇	+	+
.....	VS ₂	+	+	VS ₈	+	+
.....	VS ₃	+	+	VS ₉	.	.
.....	VS ₄	+	+	VS ₁₀	.	+
.....	VS ₅	+	+	VS ₁₁	+	+
.....	VS ₆	+	+				

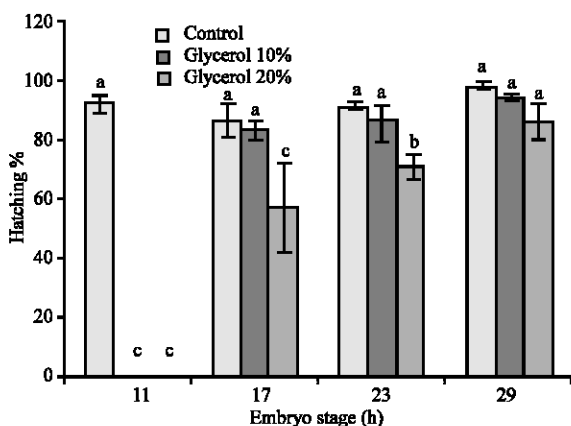


Fig. 3: Hatching rates of tench embryos following exposure to 10 and 20% glycerol at 22°C. Groups with the same superscript do not differ significantly (p < 0.05)

Concerning the results of vitrification, no viable embryos in any of the done experiments were obtained. When embryos were vitrified using VS₁-VS₆ in five steps, straws became opaque partially or entirely during cooling, showing ice formation. However, the appearance of embryos existing in transparent portions of the straws (25% of the embryos) was observed and all of the embryos became opaque during vitification, indicating that the vitrification process and penetration of vitrifying solutions to the embryos were not successful in preventing the ice formation (data not shown). Regarding vitrification of embryos in straws using VS₉ and VS₁₀, the vitrifying solutions remained transparent in all trials during cooling. However, the VS₁₀-VS₁₁ became opaque during warming.

For these experiments at which embryos were vitrified and were plunged directly in beakers with LN₂, no living, viable embryos after vitrification were obtained.

DISCUSSION

One of the main obstacles to successful cryopreservation of fish embryos is chilling injury, in which certain types of cells are damaged just by cooling

to supra-freezing temperatures, because cells must pass through chilling temperatures before being cooled at ultra-low temperatures.

In the present study, tench embryos at the 11-h stage were the most sensitive to chilling at 0 C. However, sensitivity decreased as the development proceeded and 29-h embryos were considered insensitive to chilling. The current findings are in compatible with those results recorded by Zhang and Rawson (1995) and Hagedorn *et al.* (1997) who found that zebrafish embryos at early cell division stages are sensitive to chilling, but this sensitivity decreases as development proceeds. Furthermore, early stage embryos are more sensitive to chilling than later-stage embryos in some fishes, including rainbow trout (Haga 1982), fathead minnow (Cloud *et al.*, 1988), olive flounder (Sasaki *et al.*, 1988) and medaka (Delgado *et al.*, 2005).

In contrast to our data Sasaki *et al.* (1988), Liu *et al.* (1999), Gwo *et al.* (1997) and Hagedorn *et al.* (1995) reported that later-stage embryos of red seabream, goldfish, red drum, respectively are more sensitive to chilling than some earlier stage embryos. Generally, however, embryos at early cleavage stages were the most sensitive to chilling, with exception of red drum embryos in which those at the 8-cell to morula stage are more sensitive to chilling than 2-cell embryos (Gwo *et al.*, 1995).

The sensitivity of fish embryos to chilling is attributed to the amounts of lipid components (Liu *et al.*, 1999). In fish embryos, a majority of lipid components are present in the yolk and the rest in the membranes. In zebrafish embryos, partial removal of yolk reduced sensitivity to chilling (Liu *et al.*, 1999, 2001). So perhaps tench embryos at 11 and 17 stages contain more lipids than later-stage embryos.

Our results showed that most often cryoprotectants tested were toxic to some extent to tench embryos at 22°C. However, no toxicity of methanol and glycerol has been detected at the 29 h stage embryos. The specific sensitivity of a given cryoprotectant was highly stage-dependent. The present results on the stage dependence to the toxicity of cryoprotectants toxicity are similar to those findings in flounder (Chen and Tian, 2005), zebrafish (Zhang *et al.*, 1993) and red drum (Robertson *et al.*, 1988) however, we used only 2 cryoprotectants in

our study and the order of toxicity of cryoprotectants as well as the order of sensitivity of developmental stages were different.

Our findings are also similar to the results of Zhang and Rawson (1993) who reported lower toxicity of glycerol in late-stage zebrafish embryos than in early stage embryos. The high sensitivity of the 11-h stage embryos to glycerol is in accord with the finding in zebrafish Harvey *et al.* (1983).

Cryoprotectants produce both protective and toxic effects on fish embryos. The higher the cryoprotectant concentration in the vitrifying solution, the more successful the vitrification. However, a cryoprotectant at high concentration exerts greater toxicity than the same cryoprotectant at a lower concentration which is quite obvious in the present study where high cryoprotectants concentrations, influenced the hatching rates negatively, reducing it significantly when compared with controls specially when 20% methanol was used with 11 and 23 h stages embryos and when 20% glycerol was used with 11 and 17 h stages embryos. These results are in agreement with Robles *et al.* (2003) who found that the toxicity of cryoprotectants to turbot embryos increased with the concentrations. It was not possible to ascribe the cause of toxicity in our experiments, especially since the cause could vary with the different developmental stages studied. Explanation of stage-dependent sensitivity to cryoprotectants in tench embryos requires further study.

At last, we tried to vitrify tench embryos by using nine vitrifying solutions (VS₁-VS₉) that were prepared using methanol alone, glycerol alone or combination of both in hatchery water in different proportions. Unfortunately, we could not obtain viable embryos after vitrification in any of conditions examined. In other experiments we tried to vitrify embryos using VS₉-VS₁₁ which are prepared according to modified method to that reported by Chen and Tian (2005). The latter authors obtained a high survival rate (32%) and viable embryos after vitrification using VS₉ (FVS1 in original text). However, we could not obtain viable embryos after vitrification of a total of 5500 embryos with VS₉-VS₁₁. Our results are in consistency with Edashige *et al.* (2006) who failed also to get viable embryos using Chen and Tian method.

There are many factors suspected to be the cause of the unsuccessful embryo vitrification. The first factor may be the developmental stage of embryos where we have used 23-h and 29-h stages embryos during this current study while Chen and Tian (2005) used late developmental stages of 38-h, 49-h, 67-h and 87-h embryos for their successful experiments.

The sensitivity of the embryos to the toxicity of vitrification solutions, the low permeability of embryos to water and cryoprotectants as well as the type of basic

solution that used to prepare all vitrifying solutions are the other suspected factors for unsuccessful vitrification of tench embryos.

CONCLUSION

In conclusion, it was quite difficult to vitrify the tench embryos during this study using various vitrifying solutions and the method reported by Chen and Tian (2005) and further studies using other developmental stages, basic, washing and vitrifying solutions are needed to achieve successful cryopreservation.

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