

Cryopreservation of common carp (*Cyprinus carpio*) embryos using different cryoprotectant

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Abstract

This study reports that common carp (*Cyprinus carpio*) embryos were successfully frozen and preserved by vitrification. The modified Haga solution was tested by using two different cryoprotectants and three different concentrations at 4°C to obtain appropriate vitrified solutions ((dimethylsulfoxide 5, 10, 15% (DMSO) and glycerol 5, 10, 15% (gly)) in the study. The hatching rates of embryos exposed to cryoprotectants were also evaluated. The results showed that when 2-hour stage embryos were incubated with 10 and 15% DMSO levels, no hatching was observed, while 36% rate was obtained in the 5% DMSO group. The percentage of embryos was incubated in Hank's solution (HBSS) showed a highly significant difference ($p<0.05$) compared to those were incubated in the hatchery water (Hw). The hatching rate of embryos was incubated in HBSS with 5% gly also showed a highly significant difference ($p<0.05$) than those embryos were incubated in Hw only, Hw with 5% gly, 10% gly and 15% gly and those incubated in HBSS with 15% gly. For the cryopreservation results of 49-h stage carp embryos, four embryos (8%) were recovered from 100 frozen embryos after thawing of 49-h stage embryos using extender (Haga solution) in LN2 (liquid nitrogen). At the end of the study, it was revealed that DMSO with the concentration 10% - 15% showed lethal effect on carp embryos. The study suggests that cryopreservation of carp embryos is possible and the results showed that the best cryoprotectant which was used for cryopreservation is 5% DMSO. The results of this investigation establish that cryopreservation of carp embryos is possible by vitrification. However, more studies are needed in the future to increase the carp embryos survival rate.

Keywords: Carp, Cryopreservation, Cryoprotectant, Survival, Hatching.

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Introduction

Globally, carp is the most important species of fish farming industry, both in terms of quantity and total value. According to the Food and Agriculture Organization of the United Nations (FAO), 123 countries or regions reported cultured carp production to FAO in 2018. The global culture based carp production reached 28.9 million tonnes in 2018, which is historically high (FAO, 2020).

Cryobanking of egg and embryos becomes very important subject to enhance fish stocking through aquaculture technologies and reduce the dependency on wild stock for fish production, (Diwan *et al.*, 2020). Nowadays, cryopreservation of fish sperm is carried out successfully for many fish species. However, it is not possible to mention about successful cryopreservation for fish eggs and embryos as these techniques are newer. Studies on cryopreservation of fish eggs/embryos have been carried out by many researchers, but cryopreservation has not been successful due to too many compartments in the structure of eggs and embryos (Kimmel and Law, 1985; Zhang and Rawson, 1995; Hagedorn *et al.*, 1997; Harvey, 1983; Suzuki *et al.*, 1995; Chao and Liao, 2001; Diwan *et al.*, 2010). Not only freezing but also thawing is a very important step in the cold storage process of fish gametes and embryos. Almost all vitrified solutions are currently used contain a combination of several permeable cryoprotectants and one or more impermeable frost protection. This process must take place

as soon as possible to prevent the cell membrane from crystallizing (Engin *et al.*, 2020).

Chilling studies on different fish embryos have been reported several times by Maddock (1974) for brown trout (*Salmo trutta* sp.), Haga (1982) for rainbow trout (*Onchorhynchus mykiss*), Jaoul and Roubaud (1982), Roubard *et al.* (1985), Magnus (1994), Magnus *et al.* (1996), for carp (*Cyprinus carpio*), Zhang *et al.* (1995), Hagedorn *et al.* (1997) for zebrafish (*Brachydanio rerio*), and Liu *et al.* (1993) for goldfish (*Carassius auratus*). These studies reported that the larvae were very sensitive after post-gastrulation. In addition, only a little data are available regarding the effect of cryoprotectants on fish embryos. Although it has been reported that successful freezing storage of carp embryos has been performed (Zhang *et al.*, 1989), a complete standard has not been established, yet. Recently, successful cryopreservation of zebrafish embryos using gold nanorods (GNRs) to aid the warming process has been reported (Khosla *et al.*, 2017). The success of embryonic cell cryopreservation, as well as in vitro culture, depends on the developmental stage of the fish where it is harvested. Because the development time of different fish is variable.

In this study, the toxicity of DMSO and Glycerol on carp embryos and survival rate of the fish were revealed. Modified Haga's solution (1982), were used to cryopreserve carp embryos.

Material and methods

The experiment was conducted at a commercial carp farm where is located in Mugla, Turkey. In May, eggs and sperms were taken from 3-years-old female and 5-years-old male individuals that reached gonadal maturity. Males were injected with carp pituitary (CP) at a dose 1 mg/kg body weight of fish while females were injected with CP at a dose of 0.5 mg/kg body weight of fish and after 12 h they received a second 2.5mg/kg b.w of fish. Semen from male fishes were stripped and stored at 0-2°C. The eggs were also taken from the females by stripped and kept in a damp cloth at the temperature of the cross to prevent the eggs from drying. 100 g eggs were fertilized with 1 -2 ml sperm, activation solution was added immediately for fertilization and mixed homogeneously. Then, fertilized eggs were incubated at 21°C.

Before the experiment, hatchery water and HBSS were used for the vitrification solution. In the experiments, morphologically normal carp embryos were used at various development stages (2-h, 6-h, 24-h and 49-h). 200 embryos in 10 mL of 5%, 10% and 15% DMSO and glycerol in 15 mL test tubes were placed in a 4°C water bath for 20 minutes (El-Battawy and Linhart, 2014). The embryos were then washed twice with hatchery water and were transferred to the small special incubator cages. The dead embryos and hatched fry were counted. The experiment was repeated three times using 200 embryos in each replicate. Control embryos were

similarly manipulated in hatchery water then transported to the incubator cages. HBSS was used in the study as it achieved a better hatching percentage of carp embryos (49-hour stage embryos) than HW. Modified Bock's (1992) solution was used for the cryopreservation process. The embryos cooled at 4°C / 20 min in the refrigerator were placed in 1.5 mL straw tubes and their mouths were closed automatically. Straw tubes were placed in the canister and gradually stored in the automatic freezer at -196°C for 10 minutes. Straws were then dissolved in a 40°C water bath for 7 seconds (El-Battawy and Linhart, 2014).

Means of the data acquired were evaluated three replicates. Statistical significance was assessed using multiple analysis of variance (ANOVA, Statgraphics version 5), followed by multiple comparison LSD range tests. Probability values <0.05 were considered significant. The statistical analysis was computed using SPSS software.

Results

Results are shown regarding the cooling sensitivity with/without DMSO at 4°C at different stages of development (Fig. 1).

Figure 1 shows the effect of different DMSO concentrations on the hatching rates of various embryos' stage Groups with the same superscript do not differ significantly ($p < 0.05$).

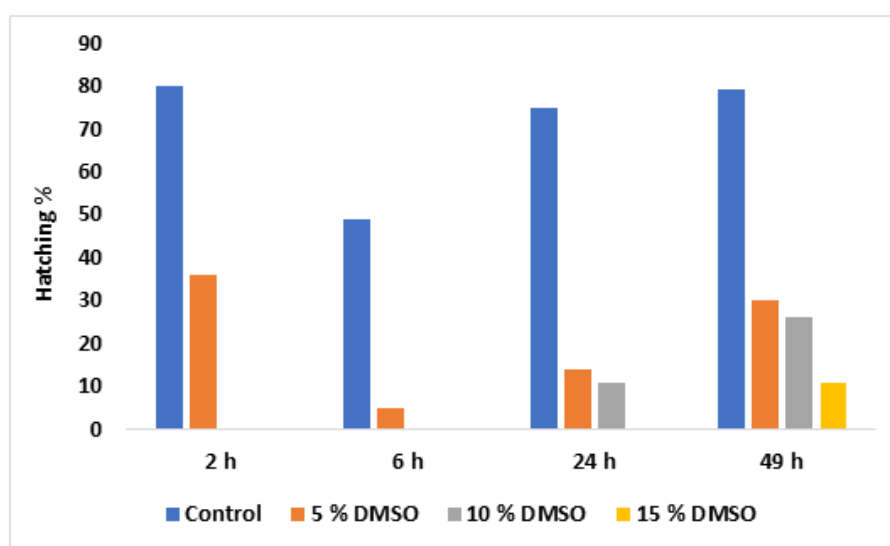


Figure 1: Effect of different DMSO concentrations on the hatching rates of various embryos' stage groups with the same superscript do not differ significantly ($p < 0.05$).

The effect of different concentrations of DMSO on the development of carp embryos was found to be significant ($p < 0.05$). While 10 and 15% of DMSO had a lethal effect on the hatching rates in the 2-hour phase, 5% DMSO showed a low significant difference compared to the control group ($p < 0.001$). There is no hatching at all three concentrations of DMSO in the 6-hour phase. In the late developmental phase (49 hours), while a significant difference was detected with the control group ($p < 0.05$), no significant difference was found between the 5% and 10% concentrations ($p > 0.05$). At the end of the study, it was revealed that embryos at the 49-hour stage were less sensitive to DMSO. On the other hand, it was determined that they were most sensitive when compared with the control group at the 2-hour stage. The effect of glycerol on the hatching rate has been demonstrated. Gly showed a protective effect and the

highest significance ($p < 0.05$) at 10% concentration found in both HBSS and HW (93.69 ± 2.38 and 88.42 ± 3.03). On the other hand, it showed low significance on the hatching rate of eggs at a concentration of 15% ($p < 0.05$) (71.43 ± 2.42 and 68.22 ± 5.45). In addition, no significant difference was found between the incubation of carp embryos in both HBSS and HW at 5% concentration ($p > 0.05$). (Fig. 2).

Figure 2 shows the impact of various Gly concentrations on the hatching rates. Groups with the same superscript do not differ significantly ($p < 0.05$).

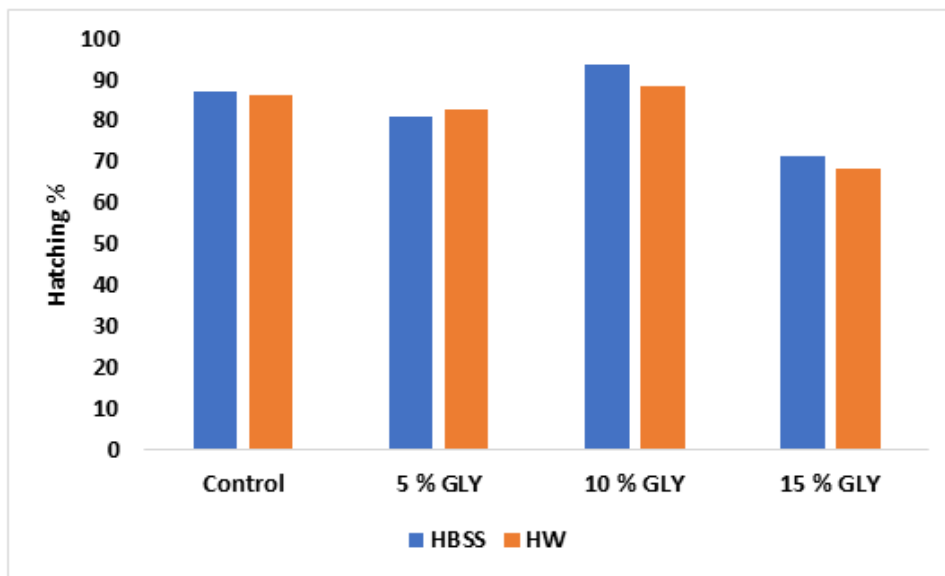


Figure 2: Shows the impact of various Gly concentrations on the hatching rates. Groups with the same superscript do not differ significantly ($p < 0.05$).

Discussion

The present study demonstrated the successful cryopreservation of carp embryos (*Cyprinus carpio*) by vitrification. When previous studies are examined, it is understood that fish egg freezing processes occur before embryo freezing. Zell (1978) reported that rainbow trout (*Oncorhynchus mykiss*) stored their eggs at -55°C , and Erdal and Graham (1980) stored at -20°C . In addition, in studies on Seaperch (*Lateolabrax japonicus*) (Tian *et al.*, 2003) and Japanese flounder (*Paralichthys olivaceus*) species, it has been reported that larvae hatching were successfully observed at the end of vitrification. On the contrary, Chen and Tian (2005) state that the protocol was not successful for Japanese flounder (*Paralichthys olivaceus*) embryos (Zhao *et al.*, 2005; Edashige *et al.*, 2006) that although hatching was successful, larvae

survival were very short. The first successful cryopreservation of fish embryos was reported in carp using the programmed slow freezing method in liquid nitrogen, but the results were not reproducible (Zhang *et al.*, 1989).

It should be noted that all CPAs have toxicity to biological systems. The relationship of the internal concentration of DMSO to fertilization and embryo survival rates in *O. lapites* embryos and the effects of various factors on these processes have been reported. DMSO passage into the cell is higher in embryos than in eggs and increased with embryonic development; however, it has been reported that the DMSO concentration in the eyed embryos reached a point in 1-5 minutes, but it did not penetrate further even if the time was prolonged (Routray *et al.*, 2002). Glycerol is the least toxic of any permeable cryoprotectants, but it is also

the least membrane permeable. Therefore, glycerol takes longer to stabilize and increases the likelihood of cryogenic injury due to reduced permeability, volume and osmotic stress (Leung and Jamieson, 1991). Despite this, the researchers stated that although glycerol has a larger molecule than DMSO, it penetrates the embryos faster, and DMSO has more toxic effects (Withler and Lim, 1982; Kerby, 1983; Harvey *et al.*, 1983; Ashwood-Smith, 1986; Riley, 2004). In this study, the hatching of embryos incubated in HBSS showed a high significant ($p < 0.05$) difference compared to those incubated in HW. In addition, the percentage of hatching of larvae incubated with glycerol was quite significant compared to DMSO ($p < 0.05$). Our results are similar to the findings of the researchers above. Also, the percentage of hatching of embryos incubated in HBSS with 10% glycerol was quite significant with the group with 5% and 15% ($p < 0.05$). The results are parallel to the findings of Zhang and Rawson (1995), who reported lower toxicity of Gly in zebrafish embryos. In addition, Gly concentration has been reported to have a significant effect on the hatching rate of turbot (*Scophthalmus maximus*) embryos (Cabrita *et al.*, 2003).

Although the cryopreservation of fish sperm has been extensively studied using teleost fish (Linhart *et al.*, 2002), there are some questions regarding the successful cryopreservation of fish eggs and embryos. Attempts have been made to preserve fish eggs but have failed due

to dehydration problems, the large size of the eggs, and the different water permeability of the membranes (Horton and Ott, 1976). In studies of fish embryos, some teleost (*C. carpio*, *Labeo rohia*, and *B. rerio*) embryos have been found to have a 100% mortality rate when stored at liquid nitrogen temperature for as little as 3 hours (Harvey *et al.*, 1983). On the other hand, Zhang *et al.* (1989) reported that widely used cryopreservation was successful for carp embryos at freezing temperatures; however, these results have not been replicated. Studies on the cryopreservation of fish eggs/embryos have been performed by a large number of researchers, but cryopreservation has not been successful in live eggs/embryos (Suzuki *et al.*, 1995; Zhang and Rawson, 1995; Hagedorn *et al.*, 1997; Zhang *et al.*, 2007; Danilo *et al.*, 2014; Keivanloo and Sudagar, 2013, 2016). At the end of the study, there are no survival embryos after thawing for cryopreservation. These results can be an association to Rall (1993) and this showed similar results with the researchers mentioned above. Rall (1993) reported that there are five main problems in the cryopreservation of eggs and embryos of teleost fish. 1. The size of fish eggs and embryos is larger than sperm cells. This situation causes severe damage after freezing and thawing, 2. The large size of the egg and embryos results in a low surface/volume ratio and lower membrane permeability, 3. Due to the presence of a multilayer membrane inhibits osmotic properties, 4. Because

of its low membrane permeability, cryo agents are difficult to penetrate and 5. fish eggs and embryos are very sensitive to sub-zero temperatures. This finding is in accord with Edashige *et al.* (2006) who reported that the Japanese flounder embryos were difficult to cryopreserve by vitrification. In the study, 8% recovery was achieved at the end of vitrification of carp embryos stored in LN2 using an automatic freezer.

Conclusion

It is apparent from the present study that using different cryoprotectants of the embryos of carp. All the eight survived carp embryos were of normal morphology and hatched successfully. However, more research needs to be done to standardize techniques and increase the effective storage times of cryopreservation fish embryos. In the future, it is necessary to increase the number of trials using different cryo agents and cryoprotectants to standardize the appropriate technique.

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