J. of Aquaculture
Vol. 19(1) : 52-56, 2006

한국양식학회지
Journal of Aquaculture
©Korean Aquaculture Society

Effects of Dimethyl-sulfoxide on Sperm Cryopreservation of Grass Carp (*Ctenopharyngodon idellus*)

Tuyet Mai Dang[1,2], Minh Anh Pham[1,2], Anh Tuan Pham[2] and Kyeong-Jun Lee[1*]
[1]*Faculty of Applied Marine Science, Cheju National University, Jeju 690-756, Korea*
[2]*Fish Genetic and Selection Department, Research Institute for Aquaculture #1, Dinh Bang, Tu Son, Bac Ninh 844-878-0614, Viet Nam*

This study was conducted to investigate the effects of three different concentrations (6%, 8% and 10% final volume) of dimethyl sulfoxide (DMSO) on cryopreserved sperm of grass carp (*Ctenopharyngodon idellus*). Grass carp sperm was suspended in Kurokura extender #2 and equilibrated at 4°C for 10 min. French straws (0.25 ml) of sperm were frozen from 4°C to -4°C at a rate of 4°C min^{-1} and then from -4°C to -80°C at a rate of 11°C min^{-1}. The straws were kept at -80°C for 10 min and finally stored in liquid nitrogen (-196°C). The cryopreserved sperm was thawed in a water bath at 40°C for 30 sec and fertilization, hatching rate and larval malformation were compared with fresh sperm (control). The fertilization rate of post-thawed sperm was comparable (from 88.21% to 94.30%) to that of fresh sperm. However, hatching rate of all frozen sperm were significantly lower ($P<0.05$) than that of control. Additionally, the larval abnormality rate of frozen sperm was significantly higher than that of fresh sperm. The results indicate that DMSO could affect the quality of cryopreserved sperm of grass carp, and a freezing program and a proper extender composition should be further studied.

Keywords: Grass carp, Dimethyl sulfoxide, Cryopreservation, *Ctenopharyngodon idellus*

Introduction

Grass carp (*Ctenopharyngodon idellus*) is a popular freshwater culture fish species in Asia due to their high growth performance and vegetable feeding habit. Recently, the interbreeding in grass carp hatcheries has been reported as a major problem leading to a genetic deterioration of broodstocks and a decline in growth of this species. The hybridization of different geographical grass carp strains is found as a feasible solution to improve the quality of broodstocks and their fingerlings. However, the transportation and/or maintenance of large number of mature fish require high costs and special facilities. Meanwhile, the cryopreservation of fish spermatozoa has been reported to be a suitable technique for transporting of fish gametes. The major advantages of the use of preserved sperm include (1) ability to maintain high quality sperm for selective and breeding programs, particularly in hermaphrodite fishes, (2) protection of stocks from transmitted diseases, (3) utilization of hatchery facilities, (4) reduction of maintenance costs for broodstocks and prevention the accidental loss of genetic sources, and (5) easiness of transportation of genetic materials (Lubzens et al., 1997). However, the cryopreservation protocol of grass carp sperm has not been established yet.

Recently, dimethyl sulfoxide (DMSO) have extensively been investigated as a cryoprotectant for several fish species, such as common carp (Kurokura et al., 1984; Babiak et al, 1997; Lubzens et al., 1997; Linhart et al., 2000; Horvath et al., 2003; Lahnsteiner et al., 2003), catfish (Steyn and Van Vuren, 1987; Bart and Dunham, 1996; Viveiros et al., 2000; Miskolczi et al., 2005), tilapia (Harvey, 1983), striped bass (He and Woods III, 2004), barramundi (Leung, 1987), mahseer (Basavaraja and Hegde, 2004), Atlantic croaker (Gwo et al., 1991), black grouper (Gwo, 1993), olive flounder (Zhang et al., 2003), winter flounder (Rideout et al., 2003), sterlet (Lahnsteiner et al., 2004) and Sakhalin taimen (Kusuda et al., 2005). The results of the previous studies have proven that DMSO could be a suitable cryoprotectant for aquatic animal sperm, but its concentration and its effects depend on species. In order to establish a consistent cryopreservation protocol for grass carp sperm, the effects of DMSO as a cryoprotectant on cryopreserved sperm of grass carp need to be studied.

Therefore, the aim of this study was to investigate the effects of different concentrations (6%, 8% and 10% final volume) of DMSO on motility, fertilization rate, hatching rate and

*Corresponding author: kjlee@cheju.ac.kr

larval malformation of frozen grass carp sperm.

Materials and Methods

Fish and gamete quality estimation

Matured grass carp were reared in the earthen ponds at Research Institute for Aquaculture #1, Dinh Bang, Tu Son, Bac Ninh, Vietnam. Fourteen mature males (1.5-2.5 kg) and 8 females (2.5-3.0 kg) were selected and kept in six glass fiber tanks (1.5 cubic meter/tank) equipped with flowing water and aeration. Spermiation in males were induced by injection of a single dose of carp pituitary extract (2.5 mg/kg body weight). Females were induced to ovulate by two injections (2.0 mg/kg initial and 8.0 mg/kg) of carp pituitary extract. Stripping for gametes collection was done after careful drying the genital pores using dry soft towels to avoid the contamination of water, mucus, feces and urine. Semen was kept individually in sterilized glass cups on ice until use (less than 1 h). Eggs from 3 females were pooled together and kept at room temperature. Cell counting was carried out as described by Linhart (1991) with some modification. Briefly, 10 μl of sperm was diluted in 990 μl of saline solution. Fifty μl of mixture was placed on the Neubauer chamber (Marienfeld, Germany) and numbered under microscopic (400X, Olympus). Motility was expressed as percentage of spermatozoa exhibiting progressive movements. Ten μl of sperm was placed on glass slide and 100 μl of water was added. The motility of sperm was observed under microscopic (100X, Olympus). The sperm with motility equal or greater than 80% were used for the next freezing trials. pH of semen was also determined.

Cryopreservation procedure

Kurokura extender #2 was prepared as described by Kurokura et al. (1984) and composed of 3.6 g NaCl, 10 g KCl, 0.22 g $CaCl_2$, 0.08 g $MgCl_2$, 0.2 g $MgCl_2$ in 1000 ml distilled water (pH 8.0). Sperm was diluted in the extender containing 3 different dimethyl sulfoxide concentrations (6%, 8% and 10% final volume) at dilution rate of 1:3 (semen:extender) in sterilized glass cups. The sperm suspensions were equilibrated for 10 min in the refrigerator at 4°C and 200 μl was drawn into 0.25 ml French straws. The freezing program was performed using Cryo-Cell CL-386 programmable freezer (USA) as described by Linhart et al. (2000) with some modification. The diluted sperm were frozen from 4°C to –4°C at a rate of 4°C per min, from –4°C to –80°C at a rate of 11°C per min, held for 10 min at –80°C and then transferred to liquid nitrogen (–196°C). The frozen sperm were thawed at 40°C for 30 sec in a water bath.

Fertilization trials

One thawed straw of sperm or 50 μl of fresh sperm was inseminated to 3 g of eggs (approximately ratio of 10^6 sperm per egg) and then activated by 5 ml of hatchery water (25°C). Three hundred inseminated eggs were randomly counted into 0.5 l plastic bowls containing 400 ml hatchery water with aeration. Water in each bowl was exchanged every 2 h. The rate of fertilization of frozen sperm and fresh sperm was checked at gastrula stage (6 h after sperm insemination). The rate of hatching and larval malformation were counted 24 h after hatching.

Statistical analysis

Data present as mean±standard deviations (S.D.). Data were subjected to one-way ANOVA in SPSS version 11.0. The significance between group means was compared using Duncan's multiple test. The percentage data of motility, fertilization, hatching and larval malformation were acrsine transformed before the ANOVA analysis. Differences were considered significant at P≤0.05.

Results and Discussion

Biological characteristics of sperm

Motility is the most common parameter to express the sperm quality, and it strongly correlates to fertilization success (Rurangwa et al., 2004). In the present study, motility of sperm was assessed after activation by hatchery water and the results are presented in Table 1. The pH, motility and density of grass carp sperm were 8.3±0.2, 87.1±7.3% and 21.0±5.5×10^9 cell/ml, respectively. The results showed that the sperm collected were suitable for preservation trials. However, the sperm motility can be initiated during the collection by con-

Table 1. Biological characteristics of spermatozoa of grass carp*

Species	n	pH	Motility of sperm (%)	Density of sperm (10^9 cell/ml)
Ctenopharyngodon idellus	14	8.3±0.2	87.1±7.3	21.0±5.5

*Data are expressed as mean±S.D.

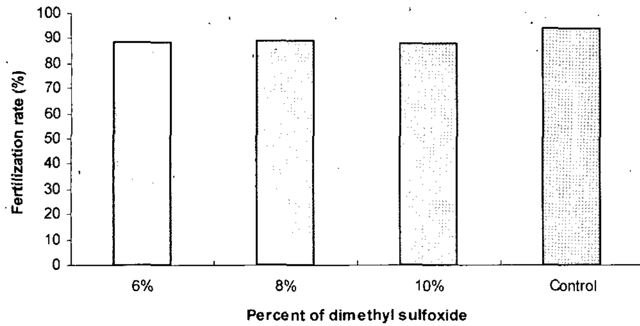

Fig. 1. Fertilization of the frozen sperm at three different concentrations of dimethyl sulfoxide.

tamination of the sperm with urine, mucus or blood; these could interfere with fertilization (Billard et al., 1995). Therefore, in present study, high quality sperm samples without any contaminant materials were used for the next trials.

Fertilization, hatching and larval malformation

The rate of fertilization of post-thawed sperm and fresh sperm is showed in Fig. 1. The rate of fertilization of frozen sperm and fresh sperm ranged from 88.21% to 94.30%, respectively. No significant differences in fertilization among all sperm samples ($P \geq 0.05$). The sperm/egg ratio (approximately 10^6 sperm/egg) applied in the present study was based on a study conducted by Bart and Dunham (1996). They concluded that ratio of 5.0×10^5 to 1.20×10^8 sperm per egg produced the highest rate of fertilization. This finding was also in an agreement of Lahnsteiner et al. (2003).

The hatching rate of grass carp sperm frozen in different dimethyl sulfoxide concentrations is presented in Fig. 2. The hatching rates of frozen sperm were significantly lower than that of the fresh sperm ($P<0.05$). However, these results were different from a finding reported by Linhart et al. (2000). They reported that fresh or frozen/thawed sperm significantly influenced the fertilization rate. However, no significant differences in hatching and larval malformation rate were found. The different males also had a significantly influence on fertilization and hatching rate. The low hatching rate of frozen sperm in the present study could be caused by some injuries during freezing process. The injuries may not affect the fertilization ability of frozen/thawed sperm, but they could lead to low survival of embryos. The fertilization of frozen sperm also can be influenced by the composition of extenders and depends on fish species. Horvath et al. (2003) demonstrated that sugar based extenders combined with 10% methanol as cryoprotectant produced higher motility, fertilization and hatching rates of frozen common carp sperm than ionic extenders in combination with 10% DMSO. However, Warnecke and Pluta (2003) concluded that modified Kurokura Extender #2 or sugar extender in combination with 15% dimethyl-acetamide final volume as internal cryoprotectant was very suitable for cryopreservation of common carp sperm, specially regard to fertilization and hatching success. In a study on cryopreservation of silver carp conducted by Alvarez et al. (2003), sperm diluted in the solution of NaCl 68.38 mM/l, sodium citrate 27.20 mM/l, and dextrose 11.01 mM/l with 10% dimethyl sulfoxide (final volume) as a cryoprotectant was frozen in nitrogen vapor for 25 min. The hatching rate of post-thawed sperm was similar to the control under farm condition, even over a year storage in liquid nitrogen. It is apparently evident that the tolerance of fish embryos to DMSO relies on species (Strussmann et al., 1999). The findings in our study suggest that grass carp embryos could be more sensitive to DMSO than silver carp. Therefore, composing a new extender for cryopreservation of grass carp sperm is recommended.

Larval malformation of cryopreserved sperm is presented in Fig. 3. The larval malformation rate of hatchlings produced by post-thawed grass carp sperm (from 4.42% in control to 9.08%

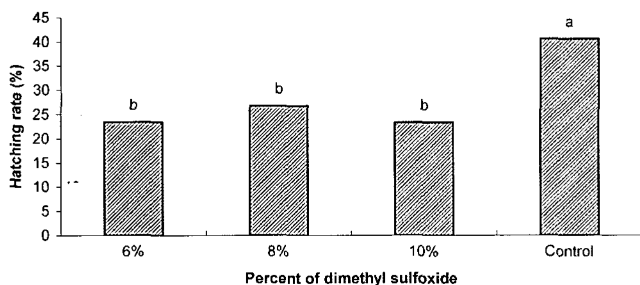

Fig. 2. Hatching rate of the frozen sperm at three different concentrations of dimethyl sulfoxide. Bars with different letters are different ($P<0.05$).

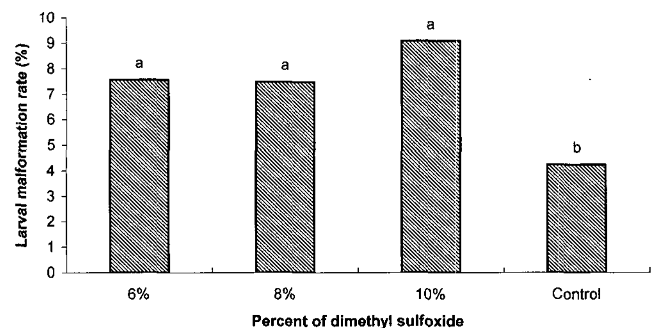

Fig. 3. The larval malformation of frozen sperm at three different concentrations of dimethyl sulfoxide. Bars with different letters are different ($P<0.05$).

in 10% DSMO, respectively) was significantly higher than that of control group. The malformation of larvae from frozen sperm might have produced by the changes of genetic materials at molecular level in frozen sperm, such as DNA (Julia et al., 2004). The changes of chromosome set in frozen/thawed sperm during freezing processes have been reported as a result to the malformation of catfish larvae. Those sperm possessing damaged chromosome set were able to fertilize eggs, but could create haploid larvae (Miskolczi et al., 2005). It is evident that the freezing/thawing processes also can affect the quality of frozen sperm (Rurangwa et al., 2004). In present study, the application of this freezing/thawing process was based on our previous trials with different freezing/thawing regimes and the current one produced highest motility, fertilization and hatching rate of frozen sperm of grass carp (unpublished data.). The results of present study suggest that higher concentration of DMSO could damage the genetic structure at molecular level and result in the malformation of hatchlings produced from cryopreverved sperm of grass carp.

In conclusion, the results of this study suggest that in order to establish a consistent cryopreservation protocol for grass carp sperm, effects of cryoprotectants, freezing/thawing programs and extender compositions should be further investigated.

Acknowledgements

This work was supported by Vietnam Ministry of Fisheries and The New Frontier Education for ECO/Friendly Marine Industry in 2005.

References

Alvarez, B., R. Fuentes, R. Pimentel, Z. Abad, E. Cabrera, E. Pimentel and A. Arenal, 2003. High fry production rates using post-thaw silver carp (*Hypophthalmichthys molitrix*) spermatozoa under farming conditions. Aquaculture, 220, 195–201.

Babiak, I., J. Glogowski, E. Brzuska, J. Szumiec and J. Adamek, 1997. Cryopreservation of sperm of common carp, *Cyprinus carpio* L. Aquacult. Res., 28, 567–571.

Bart, A.N. and R.A. Dunham, 1996. Effects of sperm concentration and egg number on fertilization efficiency with channel catfish (*Ictalurus punctatus*) eggs and blue catfish (*I. furcatus*) spermatozoa. Theriogenology, 45, 673–682.

Basavaraja, A. and S.N. Hedge, 2004. Cryopreservation of endangered mahseer (*Tor khudree*) spermatozoa: I. Effect of extender composition, cryoprotectants, dilution ratio, and storage period on post-thaw viability. Cryobiology, 49, 149–156.

Billiard, R., J. Cosson, G. Perchec and O. Linhart, 1995. Biology of sperm and artificial reproduction in carp. Aquaculture, 129, 95–112.

Gwo, J.-C., K. Strawn, M.T. Longnecker and C.R. Arnold, 1991. Cryopreservation of Atlantic croaker spermatozoa. Aquaculture, 94, 355–375.

Gwo, J.-C., 1993. Cryopreservation of black grouper (*Epinephelus malabaricus*) spermatozoa. Theriogenology, 39, 1331–1342.

Harvey, B., 1983. Cryopreservation of *Sarotherodon mossambicus* spermatozoa. Aquaculture, 32, 313–320.

He, S. and C. Woods III, 2004. Changes in motility, ultrastructure, and fertilization capacity of striped bass *Morone saxatilis* spermatozoa following cryopreservation. Aquaculture, 236, 677–686.

Horvarth, A., E, Miskolczi and B. Urbanyi, 2003. Cryopreservation of common carp sperm. Aquat. Living Resour., 16, 457–460.

Julia, K., K. Eugeny, Z. Tiantian, M.R. David and V.H. William, 2004. Effect of DNA repair inhibitor (3-aminobenzamide) on genetic stability of loach (*Misgurnus fossilis*) embryos derived from cryopreserved sperm. Theriogenology, 61, 1661–1673.

Kurokura, H., R. Hirano, M. Tomita and M. Iwahashi, 1984. Cryopreservation of carp sperm. Aquaculture, 37, 267–273.

Kusuda, S., N. Koide, H. Kawamula, T. Teranishi, J.-I. Nakajima, E. Yamaha, K. Arai and H. Ohta, 2005. Cryopreservation diluents for spermatozoa of Sakhalin taimen *Hucho perryi*. Fish. Science, 71, 293–298.

Lahnsteiner F., B. Berger and T. Weismann, 2003. Effects of media, fertilization technique, extender, straw volume, and sperm to egg ratio on hatchability of cyprinid embryos, using cryopreserved semen. Theriogenology, 60, 829–841.

Lahnsteiner, F., B. Berger, A. Horvath and B. Urbanyi, 2004. Studies on the semen biology and sperm cryopreservation in the sterlet *Acipenser ruthenus* L. Aqµacult. Res., 35, 519–528.

Leung, L. K.-P., 1987, Cryopreservation of spermatozoa of the barramundi, *Lates calcarifer* (Teleostei: Centropomidae). Aquaculture, 64, 243–247.

Linhart, O., 1991. Evaluation of the sperm and the activation and fecundity of eggs. Research Institute of Fish Culture and Hydrobiology, Vodnany, Czech Republic, pp. 1–12.

Linhart, O., M. Rodina and J. Cosson, 2000. Cryopreservation of sperm in common carp *Cyprinus carpio*: sperm motility and hatching success of embryos. Cryobiology, 41, 241–250.

Lubzens, E., N. Daube, I. Pekasky, Y. Magnus, A. Cohen, F. Yusefovich and F. Feigin, 1997. Carp (*Cyprinus carpio*) spermatozoa cryobanks-strategies in research and application. Aquaculture, 155, 13–30.

Miskolczi, E., S. Mihalffy, E. P. Varkonyi, B. Urbanyi and A. Horvath, 2005. Examination of larval malformation in African catfish *Clarias gariepinus* following fertilization with cryopreserved sperm. Aquaculture, 247, 119–125.

Rideout, R. M., K. M. Litvak and A. E. Trippel, 2003. The development of a sperm cryopreservation protocol for winter flounder *Pseudopleuronectes americanus* (Walbaum): evaluation of cryoprotectants and diluents. Aquacult. Res., 34, 653–659.

Rurangwa, E., D.E. Kime, F. Ollevier and J. P. Nash, 2004. The measurement of sperm motility and factors sperm quality in

cultured fish. Aquaculture, 234, 1–28.

Strussmann, C. A., H. Nakatsugawa, F. Takashima, M. Hasobe, T. Suzuki and R. Takai, 1999. Cryopreservation of isolated fish blastomeres: Effects of cell stage, cryoprotectants concentration, and cooling rate on postthawing survival. Cryobiology, 46, 252–261.

Styen, G. J. and J. H. J. Vuren, 1987. The fertilizing capacity of cryopreserved of sharptooth catfish (*Clarias gariepinus*) spermatozoa. Aquaculture, 63, 187–193.

Viveiros, A. T. M., N. So and J. Komen, 2000, Sperm cryopreservation of African catfish *Clarias gariepinus*: cryoprotectants, freezing rates and sperm: egg dilution ratio. Theriogenology, 54, 1395–1408.

Zhang, Y. Z., S. C. Zhang, X. Z. Liu, Y. Y. Xu, C. L. Wang, M. S. Sawant, J. Li and S. L. Chen, 2003. Cryopreservation of flounder (*Paralichthys olivaceus*) sperm with a practical methodology. Theriogenology, 60, 989–996.

Warnecke, D. and H.-J. Pluta, 2003. Motility and fertilizing of frozen/thawed common carp (*Cyprinus carpio* L.) sperm using dimethyl-acetamide as main cryoprotectant. Aquaculture, 215, 167–185.

Manuscript Received: December 12, 2005
Revision Accepted: February 7, 2006