



Effects of Different Cytoprotectants Combination on Sperm Survival, Fertility and Embryo Development in Amur Sturgeon (*Acipenser schrenckii*)

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Abstract: Development of artificial propagation technology is becoming increasingly important in sturgeon aquaculture whether species recovery efforts or commercial production. The cryopreservation technique of high-quality semen collected during the spawning season could extremely improve reproductive efficiency for year-round availability, especially for off-season use. However, the cryopreservation technique of sperm in Amur sturgeon (*Acipenser schrenckii*) has not been effectively developed. In the present study, we firstly tested the cryopreservations combination effects of three cryoprotectants, including methanol, dimethyl sulfoxide (DMSO), and propylene glycol (PG) and fresh yolk (Y) addition on sperm motility, fertilization and embryo development in Amur sturgeon. The results indicated that sperm motility was still more than 60% in the MT group but that of the control group was sharply decreased to 4.5% after 72 h of in vitro storage (4°C). The post-thawed sperm motility analysis showed that there was no significant difference between the MT+DMSO group and MT group, but the fertility rate in the MT group was significantly higher with a value of $42.30 \pm 2.57\%$ than any other experimental groups, including the MT+DMSO group ($P < 0.05$). Meanwhile, we also found that there was no significantly positive effect on post-thawed sperm motility with Y addition. Interestingly, although the results showed that the MT+DMSO group and MT group had similar effects on the post-thawed sperm motility, the MT+DMSO group had higher hatching rate compared to any other tested groups, including the MT group. Meanwhile, we also found that PG as cryoprotectant was unsuitable for sperm cryopreservation of Amur sturgeon. In conclusion, our results provides invaluable basis in further studies for the optimization technology of artificial propagation in Amur sturgeon.

Keywords: *Acipenser schrenckii*, Cryoprotectant, Sperm Cryopreservation, Embryo Development

1. Introduction

Sturgeons are polyploid chondrosteian fish that originated during the Devonian period and belong to the order Acipenseriformes, which contains 27 species divided into two families: Acipenseridae (sturgeon, 25 species) and Polyodontidae (paddlefish, two species). Sturgeon caviar is praised as “black gold” and represents one of the most highly prized foods of animal origin [1]. Therefore, wild resource of most sturgeon species has been greatly impacted by anthropogenic factors, for example, habitat destruction and

overexploitation. According to the International Union for Conservation of Nature Resource (IUCN) Red List, 85% of all sturgeon species are identified as one of the most endangered groups of animals and on the verge of extinction in the January 2018 edition (www.iucnredlist.org/). Interestingly, the conflict between the severe decline in natural populations and the huge profits from commercial sturgeon farming is the main driving force of the development of sturgeon aquaculture. Currently, more than 17 sturgeon species have been farmed and China

accounts for 15% of total sturgeon production [2]. Although the rapid development of sturgeon aquaculture has greatly eliminated the pressure on wild stocks, artificial propagation technology in sturgeon is still increasingly important for species recovery efforts as well as commercial production.

Amur sturgeon (*Acipenser schrenckii*) is a species of fish belonging to the Acipenseridae family and is found in the Amur River, Songhua River, and Heilong River [3]. Amur sturgeons are commonly used as hybridization parents of sturgeon aquaculture. However, one difficulty in hybridization reproduction is the synchronization of gamete availability from both sexes of the two different sturgeon species. Although off-season spawning can be induced by manipulating various measures, such as the temperature cycles, photoperiod, and sex-hormone stimulation, these techniques are costly and low efficiency. Moreover, the survival rate and fertility rate of sperm are often dramatically decreased in aquaculture practice. Therefore, if high-quality semen collected during the spawning season could be cryopreserved for year-round availability, especially for off-season use, this will extremely improve reproductive efficiency. However, the cryopreservation technique of sperm

in Amur sturgeon has not been effectively developed.

Vitrification cryopreservation has been recommended to increase the probability of success, and the effective preservation protocols require higher concentrations of cryoprotectants [4, 5]. To date, the methods of sperm cryopreservation have been developed in many sturgeon species, including *Acipenser baeri* [6], *A. ruthenus* [7], *Huso huso* [8] and *A. persicus* [9]. However, these studies also showed that the cryopreservation effects of different frozen extenders on sperm of sturgeon species were varied considerably. Although the frozen extenders based on methanol as cryoprotectant addition achieved the better survival rates of post-thaw sperm in many sturgeon species, the index of fertilization rate was remarkably unstable (Table 1). The latest report indicated that cryoprotectants combination were suitable for cryopreservation of sturgeon embryos [10], which might suggest that cryoprotectants combination of sperm cryopreservation in sturgeon may be more suitable method. Therefore, the present study was conducted to test the effects of different cryoprotectants combination on sperm survival, fertility and subsequent embryo development in Amur sturgeon.

Table 1. Comparison of some semen characteristics in sturgeon species following freezing-thawing process.

| Species | Extender / Cryoprotectant | Freezing procedure | Sperm motility (%) | Fertility rate (%) | references |
|---|--|---|--------------------|--------------------|------------------------|
| Siberian sturgeon (<i>A. baeri</i>) | Tris-sucrose-KCl ^a /10% MT | programmable freezer | N ^b | Over 80% | Glogowski et al., 2002 |
| Paddlefish (<i>Polyodon spathula</i>) | modified Tris-sucrose-KCl ^c /8-10% MT or DMSO | programmable freezer | 70-86% | 64-75% | Linhart et al., 2006 |
| Beluga sturgeon (<i>Huso huso</i>) | Tris-sucrose-KCl /10% MT | programmable freezer | 52-70% | 55-67% | Aramli et al., 2015 |
| Persian sturgeon (<i>A. persicus</i>) | Tris-sucrose-KCl /10% MT | programmable freezer | 60±4.1% | 72±8.36% | Aramli et al., 2016 |
| Shortnose sturgeon (<i>A. brevirostrum</i>) | modified Tris-sucrose-KCl + osmolality: 82mosM/kg /5% DMSO | 3cm-high above liquid nitrogen level, 3min; | 26±13% | 0 | Horvath et al., 2005 |
| Sterlet (<i>A. ruthenus</i>) | Tris-sucrose-KCl /10% MT | 3cm-high above liquid nitrogen level, 20min; | 5-67% | 13-76% | Dzyuba et al., 2012 |
| Amur sturgeon (<i>A. schrenckii</i>) | modified Tris-sucrose-KCl + BSA+ Glutathione /10% MT | 10cm-high above liquid nitrogen level, 5-10min; | 37.3±6.4% | 42.3±2.6% | The present study |

^a indicates Tris-sucrose-KCl (30 mM Tris, 23.4 mM sucrose, 0.25mM KCl, pH 8.0).

^b represents no statistics in the report.

^c indicates modified Tris-sucrose-KCl (20 mM Tris, 30 mM sucrose, 0.5mM KCl, pH 8.0).

MT indicates abbreviation of methanol.

2. Materials and Methods

2.1. Ethics

All experimental animal procedures followed the principles of the Guide for Care and Use of Laboratory Animals and were approved by the Animal Experimental Ethical Committee of Guangdong Institute of Applied Biological Resources.

2.2. Fish and Gamete Collection

All experiments were performed in the Engineering and Technology Center of Sturgeon Breeding and Cultivation of the Chinese Academy of Fishery Science (Beijing, China) during April and May 2016. Three adult females (IV stage; total length, 142-162 cm) and three adult males (good records;

total length, 139-158 cm) were chosen and transferred to the breeding workshop for the synchronous breeding procedure. In brief, after water temperature stimulation of 14°C -16°C for 10 day in tanks with an oxygen concentration >5 mg/L and a pH of 7.6-7.9, the parent fish received an injection of the luteinizing hormone-releasing hormone analogue (LHRH) into their pectoral fin muscle. Spermiation in males was stimulated by the LHRH dose of 2.5 µg/kg that occurred 36h before semen collection, and semen were collected with urogenital papilla by aspiration through a plastic catheter (1-2 cm diameter) connected to a 500-mL sterile plastic bottle. Sperm motility > 90% was considered to be a qualified sample for further analysis. Ovulation in females was stimulated by the LHRH at 5 µg/kg using two injection during a 12-hour interval; at the first injection, 20% of the dose was provided, and the remaining 80% provided in the second injection. The

mature degree of the egg was evaluated 24 h after the last injection.

2.3. Cryopreservation Protocol

The semen volume was measured, and sperm density was estimated using a cell haemocytometer at 200× magnification on a contrast microscope (Olympus, Tokyo, Japan). Three frequently used cryoprotectants-methanol (MT), DMSO, and propylene glycol (PG)-were used in the following experiments. The base medium (BM) of frozen extender which were mainly composed of 23.4 mM sucrose, 0.25 mM KCl, 30 mM Tris-HCl, 10 g/L BSA, 5 mM glutathione and had a pH value of 8.0, was modified according to previous methods [2]. Meanwhile, fresh yolk (Y) was also considered addition in the protocols of sperm cryopreservation for *A. schrenckii*. Therefore, we designed six groups: 1) BM + 10% MT (MT group); 2) BM + 10% MT + 10% DMSO (MT + DMSO group); 3) BM + 10% MT + 10% DMSO + 10% Y (MT + DMSO + Y group); 4) BM + 10% MT + 10% PG (MT + PG group); 5) BM + 10% MT + 10% PG + 10% Y (MT + PG+Y group); and 6) control group (fresh semen).

All the cryopreservation media were precooled overnight at 4°C of a refrigerator. Qualified semen was respectively diluted 1:1 with each of the five above-mentioned medium groups. To test toxicity of different cryoprotectants combination, the parts of each of the diluted semen of five different groups and undiluted control group were continued to be placed at 4°C of a refrigerator for further observation. In order to examine the frozen effects, majority of diluted semen were filled with 0.5-mL CBSTM cryobiostraws using micro-classic suction device (Cryo Bio System, Legler, France). Then cryobiostraws were immediately collected into CBSTM plastic cups and performed the freezing procedures, which included the following steps: first, the samples were balanced for 5-10 min on an ice-water mixture (0°C). Then, they were placed in 10 cm above liquid nitrogen for 5-10 min. Finally, the cryobiostraws were immediately immersed into liquid nitrogen (-196°C) for long-term storage.

2.4. Evaluation of Sperm Motility

To examine the frozen effects, each cryobiostraw from all the tested groups was immediately thawed for 3-5 sec in a 37°C water bath and the motility parameter was re-evaluated. Tris-HCl buffer (10 mM, PH=8.0) was used as the activating medium (AM) according to the previous method of beluga sturgeon [8]. Sperm motility was recorded by dark-field microscopy (Olympus, Tokyo, Japan). The motility percentage of each experimental group was determined on a 0-10 point scale, where 0 represented 0% motility and 10 denoted 100% motility. One person conducted all sperm motility observations to reduce the degree of variation.

2.5. Fertilization Ability and Embryo Development Assay

Equal volumes of eggs from the three females were pooled. Each group was allocated 30 g of eggs and three replicates (approx. 500 eggs / 10 g) were also designed for each group. Freshly collected and allocated eggs were immediately inseminated with post-thawed sperms from different groups. The number of fertility embryos was counted after 44 h incubation. Therefore, the fertility rate was denoted as the proportion of fertile embryos from the number of initial eggs. The number of hatched fish in each group was counted on the first day of fry emergence. The hatching rate was counted as the proportion of the number of hatched fish from the number of initial eggs.

2.6. Statistical Analyses

The mean and standard deviation (mean ± SD) were calculated from the technological and biological replication. The statistical significance among the different groups were measured using the method of one-way ANOVA with Fisher's LSD test by SPSS 17.0, with $P < 0.05$ indicating significant difference.

3. Results

3.1. The Effects of Different Cryoprotectants Combination on Sperm Motility

The properties of fresh semen from the three experimental males were evaluated as follows: semen volume 290.0 ± 14.14 (mL), sperm concentration $1.78 \pm 0.23 (\times 10^9)$ sperm / mL and sperm motility 96.3 ± 1.53 (%).

To explore the effects of different cryoprotectants combination on sperm motility at low temperature (4°C), the changes of sperm motility with storage duration times in the five tested groups were evaluated. The results showed that the effects of all five experimental groups on sperm motility were not significant after 4 h of in vitro storage. Notably, we found that the sperm motility of undiluted semen (control group) sharply reduced to 4.5% after 72 h of in vitro storage, but there was still 30.0% sperm motility even after 120 h of in vitro storage in the MT group. Meanwhile, the sperm motility of MT+DMSO groups drastically dropped to 12.3% after 24 h of in vitro storage, which might suggest that the DMSO cryoprotectant possibly inhibits sperm motility of *A. schrenckii* at low temperature (4°C). Furthermore, MT+PG and MT+PG+Y still had higher sperm motility after 72 h of in vitro storage (42.5% vs. 50.0%, respectively). The effects of the different cryoprotectants combination on sperm motility of *A. schrenckii* at low temperature (4°C) are shown in Figure 1.

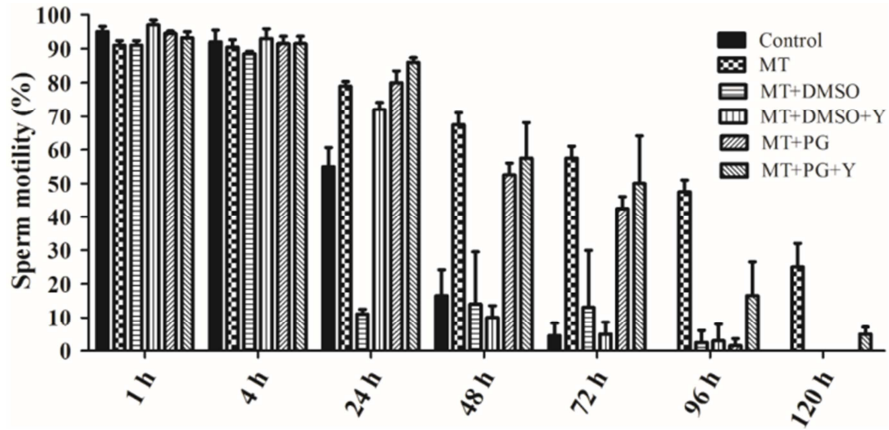


Figure 1. Effects of the different cryoprotectants combination on sperm motility (mean±SD) of *A. schrenckii*. at low temperature (4°C).

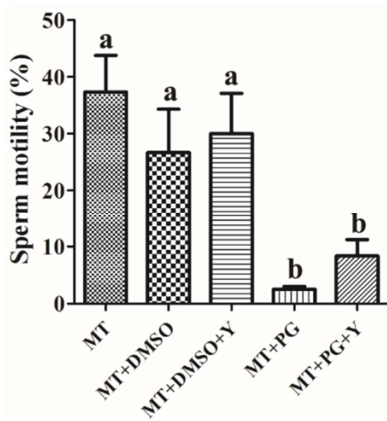


Figure 2. Effect of the different cryoprotectants combination on sperm motility of *A. schrenckii*. The different letter in each column indicates significant differences ($P < 0.05$).

More importantly, the indexes of the post-thawed sperm motility of five experimental groups were subsequently evaluated. The results indicated that the MT group had the highest post-thawed sperm motility compared to the other four experimental experiments, but the MT+PG group had the least sperm motility, only $2.5 \pm 0.5\%$. Although the two groups with fresh yolk addition (MT+DMSO+Y and MT+PG+Y) obtained

higher post-thawed sperm motility than the two groups without fresh yolk addition (MT+DMSO and MT+PG), there were no significantly positive effects on sperm motility. The information is in detail summarized in Figure 2.

3.2. The Effects of Different Cryoprotectants Combination on Fertility Ability and Embryo Development

About 32-36 h after fertilization, embryo was in yolk plug stage when animal pole was extremely bright yellow and vegetal pole was relatively dark. About 44-46 h, embryo was in the early neural stage, when neural plate began to form in the back of embryo and neural groove appeared. Subsequently, the widest part of neural plate got thicker and neural folds were formed. In the wide neurula plate formation stage, the distinct wide neural plate and U shaped nerve folds around head neural plate appeared. The development of 32-44 h embryo of *A. schrenckii* after fertilization is shown in Figure 3(a-c). Meanwhile, the embryos of different experiment groups are also observed in Figure 3(d-f), including MT group, MT+DMSO group and MT+PG group, respectively. The results showed that embryos of 44h after fertilization were mainly composed of embryos in above three development stages.

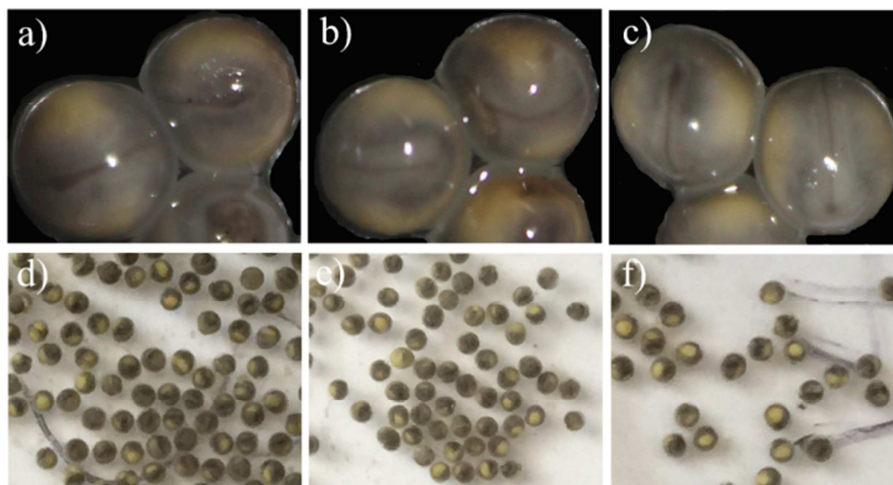


Figure 3. Development observation of 32-44 h embryos of *A. schrenckii* after fertilization. a) Yolk plug stage. b). Early neurula stage; c) Wide neural plate formation; d) Embryo of MT group; e) Embryo of MT+DMSO group; d) Embryo of MT+PG group.

The fertilization rates of the post-thawed sperm of the five experimental groups were further evaluated (Figure 4). The results indicated that the fertility rate of the MT group was significantly higher with a value of $42.30 \pm 2.57\%$, and it was followed by the MT+DMSO group. Meanwhile, the lowest fertility rate was also observed in the MT+PG group.

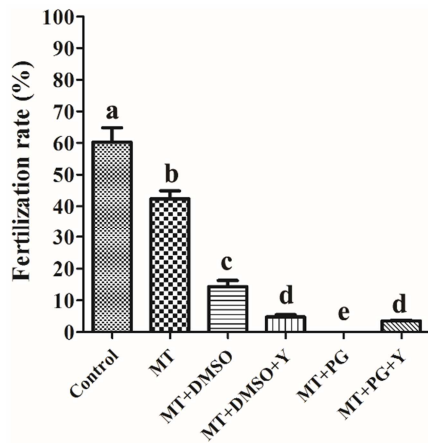


Figure 4. Effect of the different cryoprotectants combination on fertilization rate of *A. schrenckii*. The different letter in each column indicates significant differences ($P < 0.05$).

The index of hatching rate was compared among five experimental groups (Figure 5). The results indicated that the MT+DMSO group had higher hatching rate than that of the MT group, whereas the hatching rate of the MT+PG group had the significantly least effect.

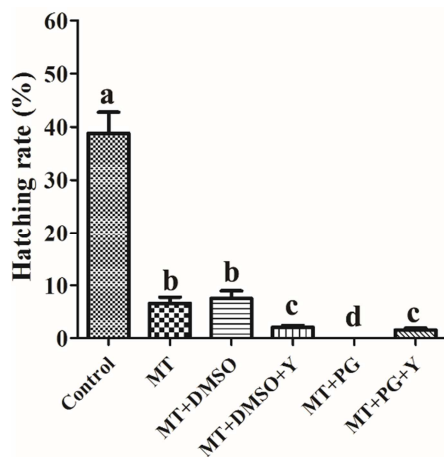


Figure 5. Effect of the different cryoprotectants combination on hatching rate of *A. schrenckii*. The different letter in each column indicates significant differences ($P < 0.05$).

4. Discussion

Sperm cryopreservation is an important technology for preserving genetic information, restoring the population of endangered species and facilitating artificial reproduction in the farming industry. To establish a successful cryopreservation technology, some essential factors need to be assessed, including the cryoprotectant agent, diluent

composition, cryoprotectant concentration, cryotube or cryotube volume, and dilution ratio [11, 12]. The damages caused by cryopreservation on fish sperm mainly occur during various processes, for example, the concentration of cryoprotectants, freezing temperature and frozen-thaw procedure; based on these factors, the selection and knowledge of the toxicity of cryoprotectant are extremely important. The objective of the present study was to evaluate the effects of cryoprotectants combination on post-thaw sperm motility, fertilization ability and embryogenesis of Amur sturgeon (*A. schrenckii*).

Sperm motility is a key index for estimating the quality of fresh as well as cryopreserved sperm [12, 13]. Usually, cryopreserved sperm has a lower percentage of motility. For example, after the cryopreservation of turbot sperm, the percentage of motile frozen-thawed sperm was significantly lower than that of fresh sperm [14]. Low motility of frozen-thawed sperm in sturgeon species is often reported (Table 1), such as the higher motile percent ($60 \pm 4.1\%$) in Persian sturgeon (*A. persicus*) [9] and the lower motility rate (only $26 \pm 13\%$) in shortnose sturgeon (*A. brevirostrum*) [15]. Recently, the Tris-sucrose-KCl system was regarded as the ideal base medium for sperm cryopreservation in Persian sturgeon [9]. Based on the Tris-sucrose-KCl system, we modified the cryopreserved medium with supplement of 10 g/L BSA and 5 mM glutamine, which have important roles in membrane protection and the prevention of sperm cell damage during cryopreservation processes. Our results showed that the sperm motility of Amur sturgeon also significantly decreased after cryopreservation, which consolidates that unavoidable cryogenic injuries occur in sturgeon during the freezing and thawing process. Meanwhile, the motility of frozen-thawed sperm in Amur sturgeon was higher than that of stellate sturgeon and shortnose sturgeon, but lower than that of Persian sturgeon, which was probably due to the different freezing methods, such as use of a programmable freezer in sperm cryopreservation of Persian sturgeon.

DMSO is the most widely used cryoprotectant in cryopreservation of fish sperm, and it provides better protection at concentrations between 5% to 25% in some marine fish species [16-18]. The embryonic development of *A. schrenckii* can be divided into nine main stages: fertilized egg, cleavage stage, blastula, gastrula, yolk plug, neurula, formation of the optic bud, heart-beat stage, and hatching stages. Especially, the stages from yolk plug to neurula formation indicates successful fertilization and are particularly important stages of embryogenesis. In the present study, after sperm cryopreservation, the MT+DMSO group obtained better effect in terms of the rate of hatched larval fish. The results is interesting and may be explained by a related report, which indicated that DMSO-based vitrificant solutions were suitable for cryopreservation of Persian sturgeon (*A. persicus*) embryos [10]. Moreover, a recent report indicated that the dramatically high level of malondialdehyde (MDA) across the embryonic development may be the main reason leading to a low hatching rate in *A. schrenckii* [19]. Probably,

we also speculate that low hatching rate in the MT group may be related with high expression level of MDA. Therefore, MT + DMSO-based method of sperm cryopreservation in Amur sturgeon is worth optimizing. For future studies, it would be desirable to assess the effect using different concentration combination of MT and DMSO as cryoprotectants on sperm cryopreservation of Amur sturgeon.

5. Conclusions

The aim of the present study was to examine cryopreservation effects of different cryoprotectants combination on sperm motility, fertilization and embryo development in *A. schrenckii*. Our results demonstrated that PG as cryoprotectant was certainly unsuitable for sperm cryopreservation of Amur sturgeon. Meanwhile, our results also showed that the post-thawed sperms can support normal embryogenesis for *A. schrenckii*, especial for high fertilization rate at the 32-44h after fertilization. More importantly, the MT+DMSO combination obtained optimal hatching edffect, which suggests that MT + DMSO-based method of sperm cryopreservation in Amur sturgeon is worth optimizing.

Abbreviations

methanol (MT), dimethyl sulfoxide (DMSO), propylene glycol (PG), the International Union for Conservation of Nature Resource (IUCN), luteinizing hormone-releasing hormone analogue (LHRH), fresh yolk (Y).

Declarations of Interest

The authors declare that they have no competing interests.

Acknowledgements

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