

Study on sperm cryopreservation of hybrid fish derived from *Carassius cuvieri* (♀) × *Carassius auratus* red var (♂)

Qianqian Zeng^a, Yixuan Chen^d, Minyi Wang^d, Yinggang Li^a, Tao Dai^a, Weiling Qin^a,
Yating Zhu^a, Chun Zhang^a, Yi Zhou^{a,e}, Qinbo Qin^{a,b,c}, Conghui Yang^{a,*}, Qianhong Gu^{a,**}

^a State Key Laboratory of Developmental Biology of Freshwater Fish, College of Life Sciences, Hunan Normal University, Changsha, 410081, Hunan, China

^b Nansha-South China Agricultural University Fishery Research Institute, Guangzhou, Guangdong, 511458, China

^c Hunan Yelu Mountain Science and Technology Co. Ltd, for Aquatic Breeding, Changsha, Hunan, 410081, China

^d Shi Cheng College, Hunan Normal University, Changsha, 410081, China

^e Hunan Hefangyu Food Technology Co., Ltd, Changsha, Hunan, 410081, China

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ABSTRACT

Cryopreservation of sperm is an effective method for conserving germplasm resources in fish genetic breeding. The high-quality hybrid fish (WR) derived from white crucian carp (*Carassius cuvieri*, WCC, ♀) and red crucian carp (*C. auratus* red var., RCC, ♂), possesses valuable traits such as high survival rates, strong resistance, and rapid growth, representing an important germplasm resources of crucian carp. This study compared the effects of different antifreeze solutions on sperm viability among three varieties (WR, WCC, and RCC) and examined changes in enzyme activity, fertilization rates, and hatching rates after cryopreservation, aiming to enhance the cryogenic sperm cryopreservation technique in hybrid fish and investigate the mechanisms underlying spermatozoa damage caused by cryopreservation. The results showed that the antifreeze combination of D14 with 15 % dimethyl sulfoxide (DMSO) had the best effect in preserving the sperm of WR and WCC, while D20 with 10 % DMSO was the optimal combination for RCC sperm. After ultra-low temperature preservation, the longevity, fertilization, and hatching rates of frozen sperm were significantly lower ($P < 0.05$) compared to fresh sperm. The enzyme activities of superoxide dismutase (SOD), lactate dehydrogenase (LDH), and creatine kinase (CK) were significantly decreased ($P < 0.05$) in spermatozoa, whereas they showed a significant increase ($P < 0.05$) in sperm plasma. Succinate dehydrogenase (SDH) activity was significantly reduced ($P < 0.05$) in frozen spermatozoa of RCC compared to fresh spermatozoa, and exhibited lower activity in frozen spermatozoa of WCC and WR. Additionally, SDH activity was significantly elevated ($P < 0.05$) in frozen sperm plasma, and glutathione reductase (GR) activity was significantly lower ($P < 0.05$) in both frozen sperm plasma and spermatozoa across all three species. The study demonstrated that cryopreservation had a significant effect on the enzyme activities of spermatozoa and sperm plasma in all three species. These findings provide important technical support for the conservation of high-quality fish germplasm resources, particularly for novel varieties resulting from distant hybridization in fish.

1. Introduction

The cryopreservation of fish spermatozoa at ultra-low temperatures has significant value in preserving valuable germplasm resources and promoting genetic breeding [1]. This technology enables the long-term preservation of fish sperm, which can effectively reduce the risk of extinction of endangered species and solve the bottleneck problem of germplasm resource degradation. Sperm from over two hundred fish

species, such as carp, salmon, and seabream, have already been effectively cryopreserved [1–9], suggesting that cryopreservation techniques for fish sperm have been widely established. Additionally, this technology can be used to establish an ultra-low temperature frozen germ cell bank. With the continuous development of fish cross-breeding technology, this approach will play a crucial role in creating new fish germplasm by addressing issues such as asynchronous development between males and females and significant differences in reproductive

* Corresponding author.

** Corresponding author.

E-mail addresses: yangch@hunnu.edu.cn (C. Yang), gqh@hunnu.edu.cn (Q. Gu).

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habits among distantly related species.

Hybridization is an effective method for preventing species degradation and creating superior varieties. In 2023, China unveiled 142 new fish varieties, with 49 of them (34.51 %) created using hybridization technology [10]. Offspring derived from distant crosses generally exhibit more pronounced changes compared to close breeding, leading to the production of superior varieties [11]. For example, hybrid F₁ derived from *Megalobrama amblycephala* (♀) × *Xenocypris davidi* Bleeker (♂), as well as the offspring obtained from backcrossing hybrids of *Megalobrama amblycephala* (♀) and *Erythroculter ilishaeformis* (♂) with the female parent, have been developed with faster growth rates and greater meat quality than their parents. In previous studies carried out by our research team, several improved fish varieties have been developed by distant hybridization, with eight of these varieties being officially approved by the Chinese government. Further work is required to improve sperm freezing and recovery techniques for these excellent germplasm resources, providing essential technological support for the breeding of new and superior germplasm resources.

However, due to the species specificity of fish sperm, the applicability of cryopreservation technology for fish sperm is limited. In order to achieve optimal long-term sperm preservation, it is essential to optimize and improve the types, concentrations, and dilutions of antifreeze tailored to specific fish varieties. The evaluation of cryopreservation effects on sperm typically includes assessment of sperm motility, viability, morphological changes, fertilizability, and changes in enzymatic activity [12]. The quality of spermatozoa can be evaluated by measuring the activities of antioxidant enzymes such as superoxide dismutase (SOD) and glutathione reductase (GR), as well as energy-metabolizing enzymes such as lactate dehydrogenase (LDH), succinate dehydrogenase (SDH), and creatine kinase (CK). These measurements have been widely used in studies involving both marine and freshwater fish [13–16].

The crucian carp plays a vital role as an economically significant freshwater fish species in China. However, its genetic resources have been severely depleted as a result of overfishing, etc. In our previous researches, we successfully obtained a high-quality hybrid fish, referred to as the WR (variety registration number: GS-02-001-2016), through distant hybridization between white crucian carp (*Carassius cuvieri*, WCC, ♀) and red crucian carp (*C. auratus red var.*, RCC, ♂) [17]. WR inherits strong resistance and fast growth rate from both parents, displaying a beautiful appearance, tender meat, and high nutritional value [18,19]. In addition, serving as a valuable genetic resource of crucian carp, WR laid the groundwork for the creation of a new fish variety known as WR-II (GS-0-001-2022). As an excellent hybrid, WR significantly contributes to enhancing the diversity of freshwater aquaculture species in China and effectively helps in mitigating the decline of crucian carp germplasm resources. In this study, cryopreservation technology was utilized to preserve the sperm of WR and its parental species (WCC and RCC). The protective properties of various combinations of antifreeze types and concentrations on semen from three fish varieties was examined using sperm viability (sperm longevity) analysis. Enzyme activities, including antioxidant enzymes such as SOD and GR, and energy metabolism enzymes such as SDH, LDH, and CK, as well as fertilization and hatching rates, were compared between fresh and frozen sperm samples. This research can provide a theoretical basis and technical support for fish distant hybridization breeding and the preservation of excellent germplasm.

2. Materials and methods

2.1. Ethics statement

Flowing the guidelines of the Administration of Affairs Concerning Animal Experimentation, approval from the Science and Technology Bureau of China and the Department of Wildlife Administration is not necessary if the fish in question are neither rare nor endangered (first- or

second-class state protection level). None of the experimental fish involved in this experiment met these criteria.

2.2. Sample collection

The fish used in this study were all from the Hunan Fish Genetic Breeding Center, China. Sperm was collected from mature male individuals of WR (175.04 ± 36.45 g, n = 8, 2–3 years old), RCC (262.90 ± 87.40 g, n = 8, 2–3 years old), and WCC (210.04 ± 45.29 g, n = 8, 2–3 years old) between April to May 2023. The fish were wrapped with a clean wet towel, dried around the genital pore with a paper towel, and then pressure was applied from the pectoral fins towards the anal fins to extract the sperm. The fresh sperm, which appeared milky white, was collected using a 50 ml centrifuge tube (sterile and non-enzyme), ensuring the sample's purity by avoiding water, blood, feces or urine contamination. Subsequently, the semen samples were stored in a refrigerator at 4 °C for preservation.

2.3. Diluent and antifreeze preparation

This study selected four dilutions (D1, D14, D5, and D20) from Table 1 to create ten combinations of antifreeze protection fluids using two antifreeze agents, dimethyl sulfoxide (DMSO) and ethylene glycol (EG), at varying concentrations (Table 2). These four dilutions were selected based on methods used in other cyprinid studies [20–22] and were validated during the preliminary experiments conducted for this study. The sperm samples from each of the three fish varieties were mixed with the ten pre-cooled antifreeze solutions at a ratio of 1:5. The mixtures were then loaded into 2 ml freezing tubes and quickly transferred to a 4 °C environment for 30 min. Afterward, the samples were placed 4 cm above the liquid nitrogen liquid phase and allowed to equilibrate for 5 min before being submerged into the liquid nitrogen liquid phase for freezing and preservation [14,23].

2.4. Sperm viability assay

After being preserved at ultra-low temperatures for 6 months, the frozen tubes were quickly retrieved from liquid nitrogen and placed in a 37 °C water bath with shaking until the ice crystals within the tubes had just melted. The thawed semen was then placed in a refrigerator at 4 °C for preservation and standby [14]. A volume of 10 µL of distilled water was pipetted onto a dry, clean microscope slide, followed by the addition of a small quantity of semen for activation in the distilled water. The samples were observed under a microscope, and sperm longevity (time from sperm activation to 90 % of sperm ceasing movement) was recorded using a timer. Repeat this procedure three times for each sample.

2.5. Enzyme activity assay

The most suitable antifreeze combination for each fish sperm was selected for the following enzyme activity test. Among the 10 antifreeze combinations tested, the combination of D14 + 15 % DMSO exhibited the most effective protection for the spermatozoa of WR and WCC, while the combination of D20 + 10 % DMSO demonstrated optimal efficacy for the spermatozoa of RCC (Fig. 1). The sperm collected from each fish were divided into two groups: (1) the control group - fresh sperm; (2) the experimental group - frozen sperm. The thawing method for the frozen

Table 1
Ingredients for the four configured dilutions.

Components (g/L)	D1	D14	D15	D20
NaCl	7.8	7	8	8
KCl	0.5	0.5	0.5	1
Glucose	15	15	15	15

Table 2
Ingredient proportions for ten antifreeze combinations.

Antifreeze solution combination	Dilution	Cryoprotectant	Dilution ratio
1	D1	15 % DMSO	17:3
2	D14	10 % DMSO	9:1
3	D14	15 % DMSO	17:3
4	D15	10 % DMSO	9:1
5	D15	15 % DMSO	17:3
6	D15	20 % DMSO	4:1
7	D15	5 % EG	19:1
8	D15	10 % EG	9:1
9	D20	10 % DMSO	9:1
10	D20	15 % DMSO	17:3

sperm samples is consistent with the procedure described above.

The samples of each fish were centrifuged at 3000 r/min for 15 min at 4 °C to separate the sperm plasma for testing. The remaining sperm were washed with a saline solution at a concentration of 9 g/L, thoroughly mixed, and then centrifuged twice under the same conditions. After the final wash, an equivalent amount of saline solution to the aspirated sperm plasma was added, mixed well, and then stored frozen at -20 °C for at least 3 h. Before the assay, the samples were naturally thawed and then centrifuged for 15 min at 3000 r/min at 4 °C. The supernatants were used to measure enzyme activities in spermatozoa [24,25]. Enzyme activity detection kits used in this experiment were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). The activities of five enzymes (SOD, LDH, SDH, GR, and CK) were compared between the sperm plasma and spermatozoa. The

respective procedures were performed according to the manual instruction.

2.6. Fertilization experiments

After 12 months of cryopreservation, we utilized fertilization experiments to assess the viability of frozen sperm of WR and WCC protected by the most effective antifreeze method. Ten mature females per varieties were randomly selected, and their eggs were gently squeezed out by pressing the abdomen and then placed in culture dishes filled with explosive water. The frozen sperm was thawed in a 37 °C water bath with shaking until the ice crystals in the cryopreservation tubes had just melted. Subsequently, the thawed sperm and eggs were mixed for insemination (at the ratio of 1×10^5 spermatozoa per egg), with fresh semen used as a control. The embryos developed in the culture dishes at a water temperature of 20–22 °C. In each group, 400 embryos (ten replicates per group) were randomly selected for assessing the fertilization rate (number of embryos at the gastrula stage/total number of eggs) and hatching rate (number of hatched fry/total number of fertilized eggs) [26].

2.7. Data analysis

The experiments on sperm viability and enzyme activity assays were conducted with three replications per group, and the resulting data were presented as mean ± standard deviation (mean ± SD). The data were organized using Excel 2016 and analyzed using STATISTICA 10 software. After testing for chi-square and normality, multiple comparisons

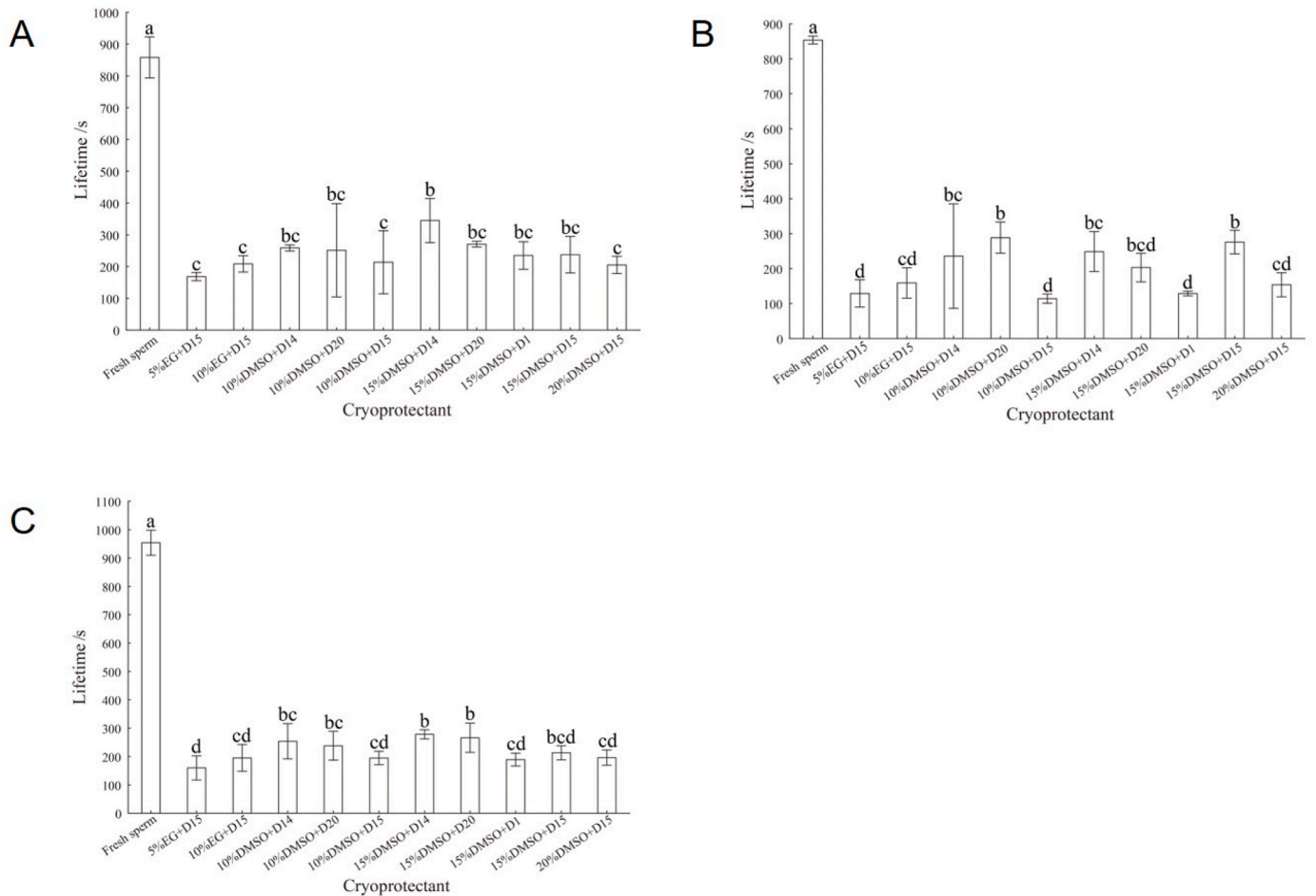


Fig. 1. Lifetime of fresh and frozen sperm of WR (A), RCC (B), and WCC (C) in different cryoprotectants. Different letters indicate significant differences between groups ($P < 0.05$), values represent means ± SD, and the following is the same.

were performed using One-Way ANOVA and Duncan's method (significant level of 0.05). An independent-samples *t*-test was used to assess the significant difference between frozen sperm and fresh sperm in the fertility test. The levels of significance were set at $P < 0.05$ for significant differences and $P < 0.001$ for highly significant differences. Finally, the results were graphed using STATISTICA 10 software.

3. Results

3.1. Effect of different antifreeze solutions on the sperm viability in the three fish varieties

The lifespans of fresh and frozen sperm in various antifreeze solutions for three varieties (WR, RCC and WCC) are shown in Fig. 1. Fresh sperm of WR lasted 858 ± 64 s, while those of RCC and WCC lasted 853 ± 11 s and 954 ± 43 s, respectively. For the frozen sperm group, the best antifreeze combination for WR and WCC frozen sperm was 15 % DMSO with D14, resulting in a lifespan of 345 ± 69 s and 278 ± 16 s, respectively. For frozen sperm of RCC, the best antifreeze combination was 10 % DMSO with D20, resulting in a lifespan of 288 ± 44 s. The longevity of frozen sperm for all three varieties in each group was significantly different from that of fresh sperm ($P < 0.05$).

Among the 10 antifreeze combinations tested, the combinations containing 5 % EG and D15 were the least effective at protecting the frozen sperm of WR and WCC, with lifespans of only 168 ± 12 s and 160 ± 42 s, respectively. For RCC, the combination containing 10 % DMSO and D15 had the shortest lifespan at 114 ± 13 s. There was a significant difference ($P < 0.05$) in antifreeze efficacy between the best and worst antifreeze solution combinations for each of the three fish varieties. Additionally, the longevity of frozen sperm was significantly different ($P < 0.05$) compared to fresh sperm across all groups of the three fish varieties.

3.2. Effect on SOD enzyme activity in sperm plasma and spermatozoa of three fish varieties

The SOD enzyme activities in the fresh sperm plasma of the three fish varieties (WR, RCC and WCC) were 10.12 ± 0.32 U/ml, 4.65 ± 0.29 U/ml and 7.68 ± 0.25 U/ml, respectively, while those in the frozen sperm plasma increased to 92.03 ± 0.47 U/ml, 77.95 ± 4.09 U/ml and 85.57 ± 1.91 U/ml, respectively. The ANOVA indicated significant differences ($P < 0.05$) in the SOD enzyme activity of sperm plasma before and after freezing for all three fish varieties (Fig. 2A).

SOD enzyme activities in fresh spermatozoa of three fish varieties (WR, RCC and WCC) were 511.95 ± 13.60 U/ml, 266.25 ± 3.53 U/ml and 340.17 ± 30.78 U/ml, respectively, while those of frozen spermatozoa decreased to 82.03 ± 5.72 U/ml, 59.95 ± 0.60 U/ml and 45.16 ± 20.17 U/ml for each variety. The ANOVA indicated significant

differences ($P < 0.05$) in the SOD enzyme activity of spermatozoa before and after freezing for all three fish varieties (Fig. 2B).

3.3. Effect on LDH enzyme activity in sperm plasma and spermatozoa of three fish species

The LDH enzyme activities in sperm plasma of fresh spermatozoa from the three fish varieties (WR, RCC and WCC) were 388.29 ± 9.67 U/L, 450.86 ± 22.36 U/L and 537.07 ± 16.10 U/L, respectively. However, the LDH activity in frozen sperm plasma increased to 11559.91 ± 16.29 U/L, 14827.96 ± 714.71 U/L and 20261.41 ± 579.20 U/L, respectively. The ANOVA indicated significant differences ($P < 0.05$) in the LDH activity of sperm plasma before and after freezing for all three fish varieties (Fig. 3A).

The LDH enzyme activities in fresh spermatozoa of three fish varieties (WR, RCC and WCC) were 7221.93 ± 1.77 U/L, 8842.64 ± 1018.90 U/L and 9475.68 ± 837.48 U/L, respectively. In contrast, the enzyme activities in frozen spermatozoa decreased to 858.30 ± 226.11 U/L, 864.18 ± 358.19 U/L and 416.39 ± 108.56 U/L, respectively. The ANOVA indicated that there existed significant differences ($P < 0.05$) in the LDH activity of spermatozoa before and after freezing for all three fish varieties (Fig. 3B).

3.4. Effect on SDH enzyme activity in sperm plasma and spermatozoa of three fish species

The SDH enzyme activities in the fresh sperm plasma of the three fish varieties (WR, RCC and WCC) were 9.50 ± 2.12 U/ml, 8.00 ± 1.41 U/ml and 6.50 ± 0.71 U/ml, respectively. Conversely, the SDH activities in frozen sperm plasma all showed an increase, with values of 168 ± 16.97 U/ml, 153.00 ± 4.24 U/ml and 150 ± 8.49 U/ml. The ANOVA indicated significant differences ($P < 0.05$) in the SDH activity of sperm plasma before and after freezing for all three fish varieties (Fig. 4A).

The SDH enzyme activities in fresh spermatozoa of three fish varieties (WR, RCC and WCC) were 85.50 ± 19.09 U/ml, 85.50 ± 2.12 U/ml and 66.50 ± 3.54 U/ml, respectively. In contrast, the SDH activity in frozen spermatozoa decreased to 63.00 ± 4.24 U/ml, 48.00 ± 8.49 U/ml and 33.00 ± 4.24 U/ml for each variety. The ANOVA indicated significant difference ($P < 0.05$) in the SDH activity of spermatozoa before and after freezing for RCC (Fig. 4B). However, for WR and WCC, there was no significant difference in SDH activity between fresh and frozen spermatozoa.

3.5. Effect on GR enzyme activity in sperm plasma and spermatozoa of three fish species

The GR enzyme activities in the fresh sperm plasma of the three fish varieties (WR, RCC and WCC) were 1133.74 ± 125.15 U/L, $359.32 \pm$

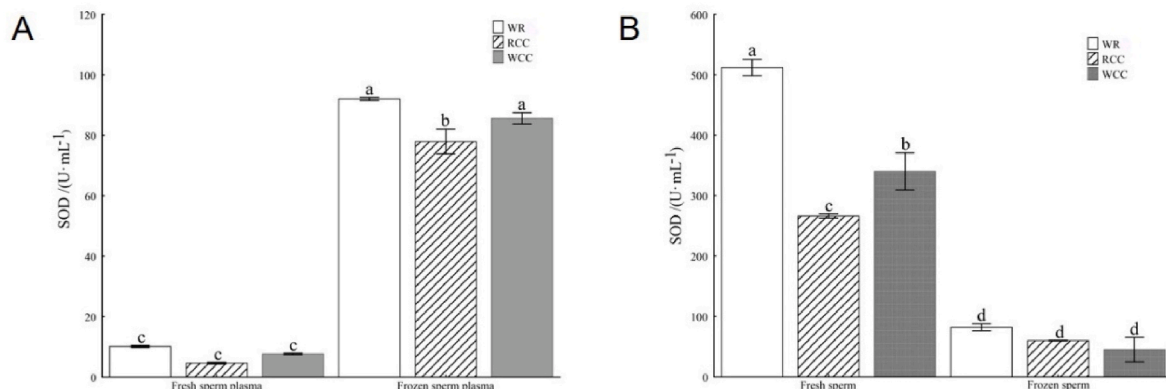


Fig. 2. Effect of ultra-low temperature cryopreservation on SOD enzyme activity in sperm plasma (A) and spermatozoa (B) of three fish varieties.

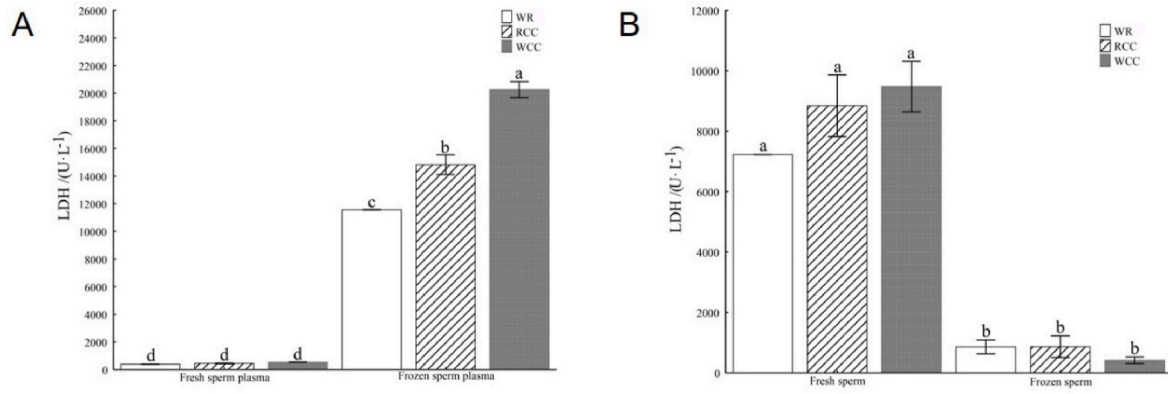


Fig. 3. Effect of ultra-low temperature cryopreservation on LDH enzyme activity in sperm plasma (A) and spermatozoa (B) of three fish varieties.

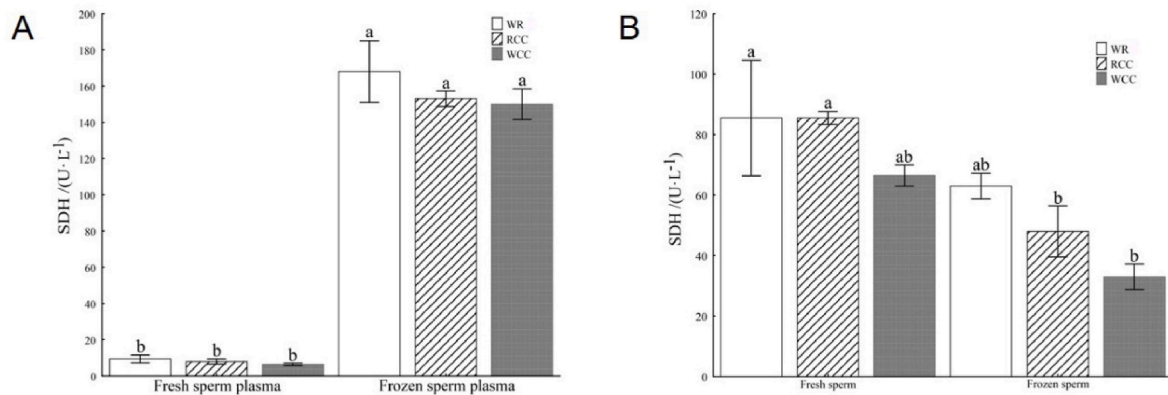


Fig. 4. Effect of ultra-low temperature cryopreservation on SDH enzyme activity in sperm plasma (A) and spermatozoa (B) of three fish varieties.

28.42 U/L and 364.95 ± 9.09 U/L, respectively. In contrast, the enzyme activities in the frozen sperm plasma decreased to 27.01 ± 5.46 U/L, 15.43 ± 5.46 U/L and 8.69 ± 1.35 U/L. The ANOVA indicated that there existed significant differences ($P < 0.05$) in the GR activity of seminal plasma before and after freezing for all three fish varieties (Fig. 5A).

The GR enzyme activities in fresh spermatozoa of three fish varieties (WR, RCC and WCC) were 355.51 ± 12.49 U/L, 50.64 ± 3.41 U/L and 53.86 ± 17.05 U/L, respectively, and those in frozen spermatozoa were decreased, which were 15.43 ± 5.46 U/L, 13.50 ± 8.19 and 7.72 ± 0 U/L. The ANOVA indicated significant differences ($P < 0.05$) in the GR activity of spermatozoa for WR and WCC (Fig. 5A), but not for RCC (Fig. 5B).

3.6. Effect on CK enzyme activity in sperm plasma and spermatozoa of three fish species

The CK enzyme activities in the fresh sperm plasma of the three fish varieties (WR, RCC and WCC) were 2.78 ± 0.15 U/ml, 2.37 ± 0.43 U/ml and 2.32 ± 0.24 U/ml, respectively, while those in the frozen sperm plasma increased to 112.89 ± 15.17U/ml, 96.47 ± 21.33 U/ml and 50.88 ± 13.75 U/ml. The ANOVA indicated that there existed significant differences ($P < 0.05$) in the CK activity of seminal plasma before and after freezing for all three fish varieties (Fig. 6A).

The CK enzyme activities in fresh spermatozoa of three fish varieties (WR, RCC and WCC) were 124.40 ± 4.32 U/ml, 114.39 ± 9.32 U/ml

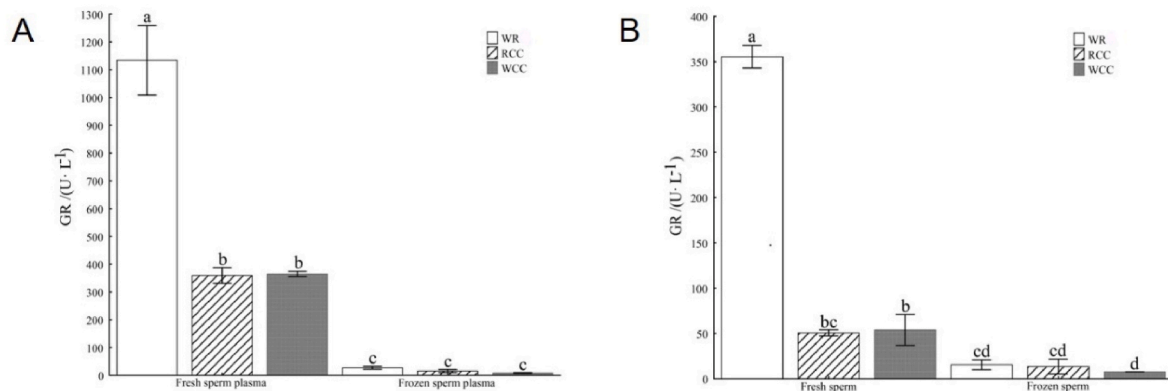


Fig. 5. Effect of ultra-low temperature cryopreservation on GR enzyme activity in sperm plasma (A) and spermatozoa (B) of three fish varieties.

and 100.09 ± 13.43 U/ml, respectively. Whereas, the enzyme activities in frozen spermatozoa decreased to 13.10 ± 4.19 U/ml, 11.62 ± 2.31 U/ml and 7.79 ± 3.48 U/ml. The ANOVA indicated significant differences ($P < 0.05$) in the CK activity of spermatozoa before and after freezing for all three fish varieties (Fig. 6B).

3.7. The fertilization and hatching rates of WCC and WR sperm

Fig. 7 displays the fertility test results of WR and WCC sperm cryopreserved for 12 months using an antifreeze solution of D14 + 15 % DMS. The fertilization rates of fresh sperm from WCC and WR were 90.31 ± 2.53 % and 86.29 ± 3.36 %, respectively, while the hatching rates were 85.62 ± 2.99 % and 82.83 ± 4.80 %, respectively. The fertilization rates of frozen sperm from WCC and WR were merely 26.11 ± 1.54 % and 26.05 ± 1.47 %, respectively, significantly lower than those of fresh sperm ($P < 0.001$). The hatching rate of frozen WCC sperm was 71.53 ± 8.46 %, displaying a significant difference compared to fresh sperm ($P < 0.001$). However, the hatching rate of frozen WR sperm (76.26 ± 3.28 %) was only slightly lower than that of fresh sperm (82.83 ± 4.80 %) ($P < 0.05$). Therefore, for hatching rates, the difference between fresh and frozen sperm appears to be less pronounced compared to the fertilization rate. The results suggest that sperm from WC and WR, after being frozen for 12 months, retained their fertilization potential, leading to successful fish hatching.

4. Discussion

4.1. Effect of different antifreeze solutions on the spermatozoa of three fish varieties

The diluent provides a suitable environment for sperm survival, prolonging their lifespan in vitro, and reducing the damage to sperm structure caused by freezing. When the antifreeze interacts with water molecules, the increased viscosity of the antifreeze solutions reduces the freezing point for ice crystal formation both inside and outside the cell, thereby preventing the sperm from ice crystal damage and achieving the purpose of antifreeze [25]. Currently, methanol (MeOH), propylene glycol (PG), EG, and DMSO are often used as antifreeze agents in the cryopreservation of fish sperm. DMSO and EG were mainly used in the cryopreservation of cyprinid fish [27–29]. Various dilutions, including Hank’s salt solution, D1, D14, D17, D15 and Ringer’s solution, have been experimented for the cryopreservation of many cyprinid fish. D1 achieved the best results in the ultra-low temperature preservation of *Coreius guichenoti* spermatozoa [7,20]. D15 was found to be more effective in cryopreserving spermatozoa from silver carp, common carp, blunt snout bream, grass carp and mandarin fish [1,30]. The study found that D14 was the most effective dilution in preserving the spermatozoa of WR and WCC, with frozen spermatozoa lifetimes reaching 345 s and

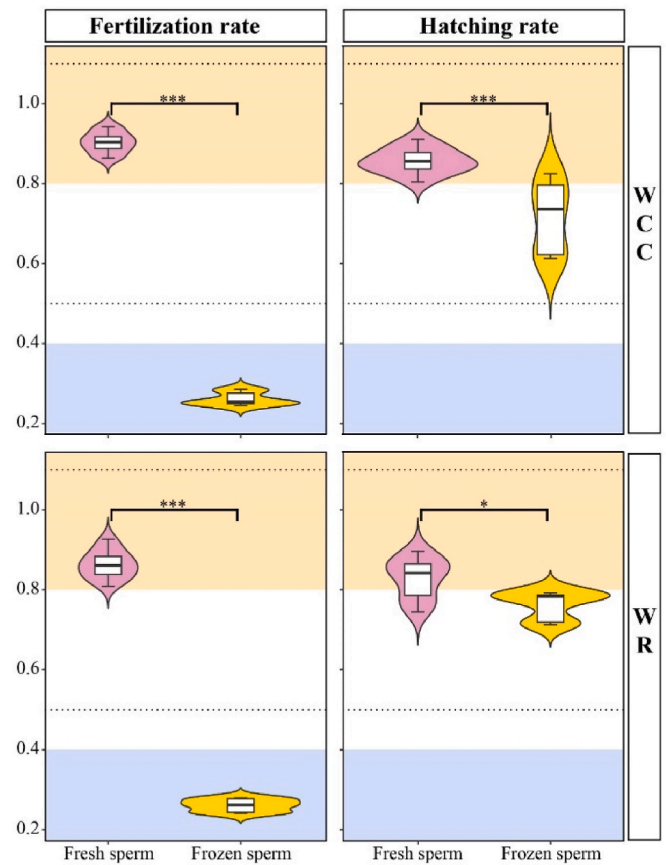


Fig. 7. Fertilization and hatching rate of fresh sperm and frozen sperm of WCC and WR after ultra-low temperature cryopreservation for 12 months.

278 s, respectively. Meanwhile, D20 was the best for preserving RCC spermatozoa, with a maximum lifetime of frozen spermatozoa of 288 s. DMSO was found to have a better antifreeze effect than EG in all cases. As the toxicity of DMSO to spermatozoa strongly correlated with its concentration, typically ranging from 5 % to 15 % [29], the present study confirmed that a 15 % DMSO concentration was most effective for cryopreserving WR and WCC sperm, while 10 % DMSO was optimal for RCC sperm.

4.2. Effect of ultra-low temperature storage on SOD and GR of sperm plasma and spermatozoa in three fish varieties

SOD and GR are two pivotal antioxidant enzymes responsible for

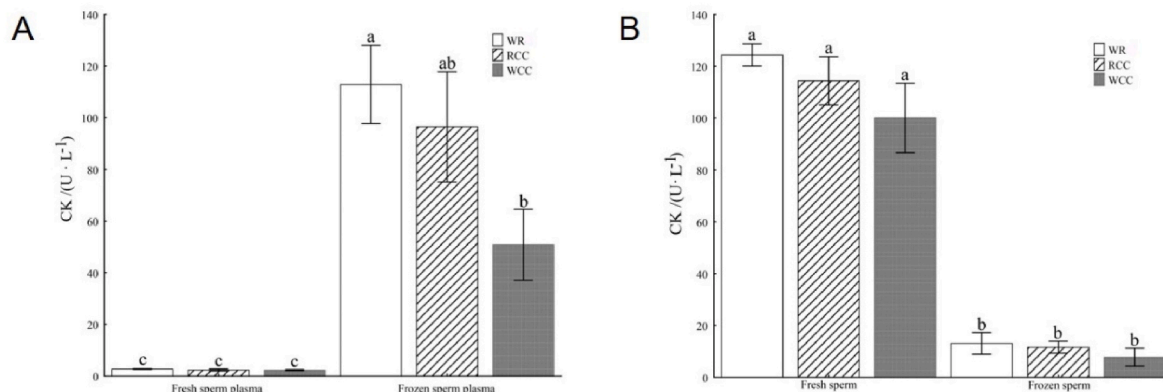


Fig. 6. Effect of ultra-low temperature cryopreservation on CK enzyme activity in sperm plasma (A) and spermatozoa (B) of three fish varieties.

scavenging oxygen-free radicals in spermatozoa, constituting crucial components of the sperm's antioxidant defense system. The levels of SOD and GR activities can be used as indicators for evaluating the quality of frozen spermatozoa [31]. SOD functions to cleanse the membrane of unsaturated lipids and protect the cell structure from damage, playing a crucial role in maintaining the oxidative and antioxidant equilibrium within the organism [32]. This study found a notable increase in SOD activity in the frozen sperm plasma of all three fish varieties, while a significant decrease was observed in spermatozoa post-cryopreservation. These results are consistent with previous studies on cryopreserved sperm from other fish species, such as *Epinephelus auratus*, *Acipenser gueldenstaedti* and *Myxocyprinus asiaticus* [6,14,24]. The ultra-low temperature storage might have caused a lipid oxidation reaction, leading to the damage of sperm membranes. As a result, SOD leaked from the spermatozoa into the seminal plasma, therefore reducing sperm viability.

GR can reduce oxidized glutathione (GSSG) to its reduced form, GSH, which is necessary to maintain glutathione peroxidase (GSH-PX) activity. Therefore, an increase in GR activity can result in a corresponding elevation in GSH-PX activity. The increased GSH-PX activity is a positive response to heightened reactive oxygen levels, contributing to remove excess reactive oxygen from cells [33]. Ding et al. [14] found that GR activity increased in cryopreserved spermatozoa of *Epinephelus auratus*, while there was no significant difference in sperm plasma. Similarly, Zhou et al. [34] observed an increase in GR activity in frozen sperm of *Acipenser baerii*. In contrast, this study found that the activity of GR decreased in both frozen spermatozoa and sperm plasma across all three fish varieties. It is possible that the enzyme structure was damaged during the process of ultra-low temperature storage due to rapid cooling rates and subsequent formation of ice crystals. Consequently, future studies should consider utilizing gradient cooling techniques and extending the equilibrium duration to enhance experimental procedures. In addition, further investigation should be conducted to determine how the structure of GR in spermatozoa changed during ultra-low temperature storage.

4.3. Effect of ultra-low temperature storage on LDH, SDH and CK of sperm plasma and spermatozoa in three fish varieties

LDH is an essential enzyme in glycolysis. SDH is a mitochondrial enzyme marker, reflecting the level of energy metabolism activity in spermatozoa [13]. CK is directly related to intracellular energy processes and ATP regeneration [35]. Therefore, all three enzymes are related to energy metabolism, and their activity levels can partially indicate sperm viability. This study found a significant increase in LDH and CK activities in the frozen sperm plasma of all three fish varieties, with a notable decrease in activity observed in the frozen spermatozoa, consistent with findings in *Acipenser gueldenstaedti* [24]. SDH activity significantly increased in the frozen sperm plasma of all three fish varieties, while it significantly decreased only in the frozen spermatozoa of RCC. In frozen spermatozoa of WCC and WR, SDH activities generally decreased without statistical significance. These findings were consistent with the decrease in SDH activity observed in frozen spermatozoa of *Acrossocheilus fasciatus* [23], *Epinephelus lanceolatus* [14] and *Myxocyprinus asiaticus* [6]. The low temperature might have destroyed the mitochondrial membrane structure in spermatozoa, leading to the loss of LDH, CK and SDH activity. Additionally, the freezing and thawing process might have caused irreversible mechanical damage to the sperm cell membrane, resulting in the release of enzymes from spermatozoa into the sperm plasma and an increase in enzyme activity in the plasma.

Following cryopreservation, the activity of antioxidant enzymes (SOD and GR) and energy-metabolizing enzymes (LDH, CK and SDH) decreased in the spermatozoa of all three fish varieties, suggesting

damage caused by sperm cryopreservation. During the process of freezing and thawing, mechanical damage to the sperm membrane occurs due to the formation and disappearance of ice crystals. Consequently, enzymes within the sperm cells are released into the seminal plasma, resulting in decreased enzyme activity in the sperm and increased activity in the seminal plasma, which reduces sperm viability and fertilization capacity [36]. Our results showed that ultra-low temperature storage caused cryo-damage to the spermatozoa of RCC, WCC and WR, which should be further investigated on the mechanisms of sperm cryopreservation damage, such as the cold shock, ice formation, cryoprotectant toxicity and osmotic effect. In addition, future work should focus on finding more suitable diluents and cryoprotectants for freezing, optimizing equilibrium time, and refining thawing methods to effectively reduce the detrimental effect of ultra-low temperature storage on spermatozoa.

4.4. Comparison on sperm enzyme activity between distant cross offspring and parents

After analyzing the sperm cryopreservation parameters of WR and its parental species (WCC and RCC), it was found that the optimal anti-freeze combination for WR sperm was identical to that of its female parent, WCC, but differed from that of its male parent, RCC. In the enzyme activity assay, discrepancies were observed among the three species. Specifically, in the frozen sperm plasma of WR, the SOD activity mirrored that of WCC, while the CK activity aligned with RCC. Additionally, there were numerous enzyme activity test results in WR sperm that differed from both the male and female parent. For instance, in fresh spermatozoa, WR exhibited higher activities of the antioxidant enzymes SOD and GR compared to the parental species. Following cryopreservation, WR displayed notably higher SDH enzyme activity in spermatozoa and significantly lower LDH enzyme activity in sperm plasma, indicating a reduced degree of damage compared to the parental species. While these findings do not conclusively indicate whether the internal environment of the hybrid offspring's sperm cells tends to inherit from the male or female parent, they do partially suggest the hybrid advantage of WR.

4.5. Effect of ultra-low temperature storage on the fertilization and hatching rates of WCC and WR sperm

Despite significant advancements in research on ultra-low temperature preservation of fish sperm, achieving fertilization rates comparable to fresh sperm with frozen sperm remains challenging. During the cryopreservation process, sperm cells sustain inevitable irreversible damage, which leads to lower viability of frozen sperm when compared to fresh sperm [37]. Factors such as rapid temperature reduction leading to the formation and growth of intracellular ice crystals, osmotic imbalances between the cell's internal and external environments resulting in dehydration and deformation of sperm cells, and cytotoxicity of permeable antifreeze agents contribute to this damage [38]. In addition, in this study, the decreased fertilization rate of frozen sperm may be linked to prolonged ultra-low temperature storage, as the metabolic activity of sperm cells does not completely cease at -196°C in liquid nitrogen, resulting in decreased sperm viability over extended storage periods. Research on red seabream (*Pagrus major*) sperm revealed that sperm motility and fertilization rates were notably lower in samples cryopreserved for 360 days compared to those stored for <60 days [39]. Similar findings have been reported in studies on sperm cryopreservation of common carp (*Cyprinus carpio*) [40] and sharpooth catfish (*Clarias gariepinus*) [41]. Additionally, the sperm-egg ratio is crucial for fertilization and hatching rates. Studies have shown that the fertilization rate of frozen sperm increases with higher sperm-egg ratios [42,43].

Using frozen sperm quantities exceeding fresh sperm by tenfold or even several tens of times for fertilizing a certain number of eggs can lead to high fertilization rates even with low sperm viability. Furthermore, the difference in hatching rates between frozen and fresh sperm of WR and WCC was not as significant as that in fertilization rates, indicating that the primary requirement for frozen sperm is to produce fertilized eggs, because embryos produced from frozen sperm have a high probability of producing viable offspring as long as they can develop normally through the blastocyst stage. Therefore, in future studies, improving the cryopreservation technique of hybrid fish and their parental sperm may be achieved through increasing the ratio of sperm to egg in fertilization.

5. Conclusions

The study screened the sperm cryopreservation parameters of the hybrid fish WR and its parental species (WCC and RCC), and identified the optimal antifreeze combinations: D14 as a diluent and 15 % DMSO as a cryoprotectant for WR and WCC, and D20 with 10 % DMSO for RCC. Although cryo-damage resulting from ultra-low temperature storage significantly reduces sperm longevity, fertilization and hatching rates, as well as alters enzyme activities in spermatozoa and sperm plasma, the study successfully established a preliminary sperm cryopreservation method for the three fish varieties, which offers technical support for the long-term conservation of crucian carp germplasm resources.

CRedit authorship contribution statement

Qianqian Zeng: Writing – review & editing, Writing – original draft, Investigation. **Yixuan Chen:** Investigation. **Minyi Wang:** Investigation. **Yinggang Li:** Investigation. **Tao Dai:** Investigation. **Weiling Qin:** Investigation. **Yating Zhu:** Investigation. **Chun Zhang:** Resources, Investigation. **Yi Zhou:** Resources, Investigation. **Qinbo Qin:** Resources, Investigation. **Conghui Yang:** Writing – review & editing, Writing – original draft, Methodology, Funding acquisition, Conceptualization. **Qianhong Gu:** Writing – review & editing, Writing – original draft, Methodology, Funding acquisition, Conceptualization.

Declaration of competing interest

Chun Zhang, Yi Zhou, and Qinbo Qin are Editorial Board Members for Reproduction and Breeding and were not involved in the editorial review or the decision to publish this article.

All authors declare that there are no competing interests.

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