Genetics of Aquatic Organisms 7(4. Special Issue), GA616
https://doi.org/10.4194/GA616

RESEARCH PAPER

Evaluation of Extenders for Cryopreservation of *Cirrhinus mrigala* Milt

Insherah Aziz[1], Shaista Bibi[1,2], Bushra Allah Rakha[1], Sobia Amir[1], Muhammad Amjad Awan[1], Javeria Arshad [1], Shamim Akhter[1,*]

[1]Department of Zoology, Wildlife and Fisheries, Pir Mehr Ali Shah Arid Agriculture University Rawalpindi-46300, Pakistan
[2]Department of Fisheries, Government of Punjab, Pakistan.

How to Cite

Aziz, I., Bibi, S., Rakha, B.A., Amir, S., Awan, M.A., Arshad, J., Akhter, S. (2023). Evaluation of Extenders for Cryopreservation of *Cirrhinus mrigala* Milt. *Genetics of Aquatic Organisms, 7(4. Special Issue), GA616*. https://doi.org/10.4194/GA616

Article History
Received 08 July 2023
Accepted 20 October 2023
First Online 15 November 2023

Corresponding Author
E-mail: sashraf1993@gmail.com

Keywords
Kurokura-2
Alsever's solution
DMSO
Methanol
Milt

Abstract

This study aimed to evaluate the performance of different extenders for the cryopreservation of *Cirrhinus mrigala* milt. The extenders under investigation included Kurokura-2 + DMSO (KD), Alsever's solution + DMSO (AD), Kurokura-2 + Methanol (KM), and Alsever's solution + Methanol (AM). Sperm quality assessments encompassed critical parameters such as sperm motility, motility duration, viability, and DNA integrity. The initial analysis of fresh milt revealed motility rate of 88.33±1.66%, pH of 7.52±0.76, a volume of 7.83±0.44 mL, a sperm count of 3.55±0.40 x 10^{10}/mL. Upon thawing, it became evident that the KD extender yielded significantly higher post-thaw sperm motility (%) at 68±1.67, compared to the KM (40.00±2.89), AD (38±1.67), and AM (41.67±4.40). Additionally, sperm motility duration (seconds) exhibited a notable increase in the KD extender (78.67±10.413) in comparison to the KM (52.00±5.291), AD (46.66±1.66), and AM (41.67±1.667) extenders. The KD extender also contributed to higher sperm viability (%) at 66.00±1.00, whereas KM and AM exhibited similar values at 59.00±3.00 and 55.33±6.38, respectively. Furthermore, DNA integrity (%) remained consistently high for KD, KM, and AM (98.67±0.66, 98.00±1.52%, and 97.33±1.20%), while a lower percentage was observed for AD (88.00±1.15). In conclusion, the modified Kurokura extender, supplemented with 10% DMSO, showcased a pronounced positive impact on the cryopreservation of *Cirrhinus mrigala* milt.

Introduction

Cirrhinus mrigala, a vital component of pond polyculture systems in Pakistan, India, and Bangladesh, shares its aquatic habitat with fellow species such as *Catla catla*, *Cyprinus carpio*, and *Labeo rohita* and thrives as a benthic feeder. It diligently consumes detritus and leftover surface feed, effectively contributing to water resource management and efficient feed utilization. Further, owing to its meat quality, excellent taste and relatively fast growth, this species has gained much interest of aqua-culturists globally (Haider et al., 2018).

Cirrhinus mrigala is a seasonal breeder and spawns naturally from June to September. Gonadal of maturation *Cirrhinus mrigala* is triggered by low water temperatures, increased rainfall and sufficient nutrition (Routray et al., 2007). In hatcheries, natural stimulatory conditions are nearly impossible to be maintained and the fish are induced to spawn (Rottmann et al., 1991) using synthetic hormone also for synchronized spawning of male and female (Naik & Mirza, 1992). Hatcheries acquire production only by means of a small number of brood stock. Therefore, the hatchery carps are bred repeatedly among themselves and chances of cross breeding remain almost nil. This scenario is also

responsible for inducing genetic drift by eradicating many alleles from populations resulting in detrimental effects on reproductive capacity and physiological efficiency of fish (Eknath & Doyle, 1990).

Milt cryopreservation technique assists aquaculturists to overcome the consequences of inbreeding as it allows easy transportation of milt to be used in selective breeding programs for genetic improvement of brood stocks, reducing inbreeding. In addition, cryopreserved spermatozoa can be used for *in-vitro* fertilization in case of asynchronous maturation of males and females. Also, the number of male brooders maintained at hatchery can be reduced (Thommai & Devi, 2016). Moreover, in case of disease outbreaks, overexploitation and natural disaster, cryopreservation can help in stock protection from being totally eliminated (Agarwal, 2011).

Milt is diluted with suitable medium/extender (Stoss, 1983) that did not infer motility and keep the sperm inactive (Routray & Verma, 2011). It is a formulated buffered physiological saline solution with respect to the inorganic composition of seminal plasma, so its composition is standardized for different species (Borchard & Schmidt, 1979). Kurokura-2 and Alsever's solution have been used for milt cryopreservation different cyprinid species. Cryoprotectants are constituents of extenders that enhance the cryo-resistance of the sperm and accord different protective effects in different species (Yavaş et al., 2014). Cryoprotectants such as DMSO, methanol, glycerol, propanediol are reported to decrease the freezing point of the solution, inhibit the intracellular ice crystal formation and reduce osmotic shock (Leung, 1991).

Present study was conducted to characterize milt (motility, volume, pH, concentration) and evaluate extenders (Alsever's solution and modified Kurokura extender) in combination with cryoprotectants (DMSO and Methanol) for cryosurvivability of *Cirrhinus mrigala* milt.

Materials and Methods

Collection of Milt

Milt was collected from mature male brooders (n=3) of *Cirrhinus mrigala* weighing 1.5-2 kg, reared at Fish Seed Hatchery Rawal Town, Islamabad. Milt was collected in graduated falcon tubes carefully to prevent contamination of fecal material and other body fluids like urine and blood. Milt was transferred to Animal Physiology Laboratory, PMAS AAUR, at 4ºC for further analysis and processing.

Estimation of Milt Color, pH, Volume, Motility, Sperm Count

The success of cryopreservation primarily relies on the quality of fresh milt. Parameters used to assess its quality include color, volume, pH, motility, and sperm count.(Agarwal, 1994). Milt of creamy white color was selected for further processing as it indicates that it is free of contamination. pH of the milt was measured using pH meter (Multi meter Real Time Data logger, YK-2005WA, Lutron). Milt volume was measured using graduated falcon tubes. Sperm motility was accessed by diluting milt with distilled water and expressed as percentage or forward moving spermatozoa and assessed using Celestron microscope (Model#44345) at 40X. Spermatozoa counting was done by adding a 5-6ul drop of 1000 times diluted milt with physiological saline solution onto hemocytometer slide and observed under an inverted microscope. Sperm concentration was expressed as number/mL (Gupta et al., 1993).

Extender Preparation:

Kurokura-2 Extender (Magyary et al., 2000) and Alsever's Solution (Das et al., 2010) were prepared and cryoprotectants (DMSO and methanol) were added at 10% to make four types of extenders as shown in the Table 1.

Table no. 1: Chemical composition of extenders used for cryopreservation of *C. mrigala* milt.

Components	Extenders (100 mL)			
	E_1	E_2	E_3	E_4
NaCl	360mg	360mg	0.4%	0.4%
KCl	1000mg	1000mg		
$CaCl_2$	22mg	22mg		
$MgCl_2$	8mg	8mg		
$NaHCO_3$	20mg	20mg		
Sodium Citrate			0.8%	0.8%
DMSO	10%		10%	
Methanol		10%		10%

*Four extender/Treatments shown in Table 1
E1= Kurokura-2 +10%DMSO (KD)
E2= Kurokura-2 +10%Methanol (KM)
E3= Alsever's solution +10%DMSO (AD)
E4= Alsever's solution+10%Methanol (AM)

The milt was diluted with extender in the ration of 1:9. Milt diluted with extender having methanol was frozen immediately after dilution while milt diluted in extender having DMSO was given 10 minutes for equilibration (Horváth et al., 2003). The milt was then filled into straws (0.5 ml) and sealed using sealing powder. After sealing, straws were subjected to vapor of liquid nitrogen for 3 minutes at the height of 3 cm and then immersed into liquid nitrogen and kept for 7 days.

Post Thaw Analysis

The samples were thawed at 25^{o}C for 30 seconds in a water bath and further analyzed for sperm motility, motility duration, viability and DNA integrity.

Sperm Motility and Motility Duration: The motility of chilled milt was observed using inverted microscope (Celestron; X400) immediately after dilution with sterile water on glass slide. Percentage motility of the sperm was estimated at 0-30 seconds after activation. Duration of sperm motility was settled as time when sperm was activated until stopped moving (Akçay et al., 2004).

Sperm Viability: Eosin-nigrosin staining was used to study the viability of spermatozoa. Milt (3µl) was mixed on a clean glass slide with both eosin and nigrosine (9µl) respectively, and then the mixture was smudged on another glass slide. After that slide was examined under light microscope at magnification of x1000. Live sperm were colorless while dead sperm appeared pink (Kledmancee et al., 2013). A total of 100 spermatozoa were counted and viability of sperm was expressed as percentage.

DNA Integrity: DNA integrity of sperm was assessed through acridine orange test as described by (Awan et al., 2021) with slight modifications in staining and washing steps. Thin smear was prepared on a glass slide, air dried and fixed in Carnoy's solution (100ml; glacial acetic acid 75ml, Methanol 25ml). After 2 hours, slides were removed from Carnoy's solution and air dried. Then for 5 minutes these fixed slides were placed in Tampon's solution (15 mmol/L $Na_2HPO_4 \bullet 7H_20$; 80 mmol/L citric acid) at 75 degrees Celsius. Slides were stained for 20 seconds with 0.02% acridine orange, washed three times with distilled water and analyzed under epifluorescence microscope (Labomed LX400) at 400X Yellow or red fluorescence was emitted by sperm containing damaged DNA and green fluorescence was emitted by sperm with intact DNA (Awan et al., 2021).

Experimental Design and Statistical Analysis

Four experimental extenders (treatments) were investigated for cryopreservation of *Cirrhinus mrigala* sperm. The experiment was repeated three times (replicates). In each replicate, milt was collected from three male brooders, pooled and aliquoted for dilution in experimental extenders. Treatment effects were analyzed using Analysis of Variance (ANOVA). Means were compared by Duncan's Multiple Range Test and a value of $p<0.05$ was considered as being statistically significant. Data were expressed as mean ± SE.

Results

Fresh Milt Characteristics

Cirrhinus mrigala milt, free from contamination was creamy white in color. Milt pH was 7.52±0.76 with average total volume of 7.83±0.44 mL, sperm count of 3.55±0.40 x10^{10}/mL and the motility up to 88.33±1.66%. Milt samples that fulfilled above mentioned criteria were selected for further processing for cryopreservation.

Post Thaw Sperm Quality Parameters

Sperm Motility

The data on the impact of different extenders on post-thaw motility (%) of *Cirrhinus mrigala* sperm is summarized in (Figure 1). In present study post thaw sperm motility was higher in modified Kurokura-1 +

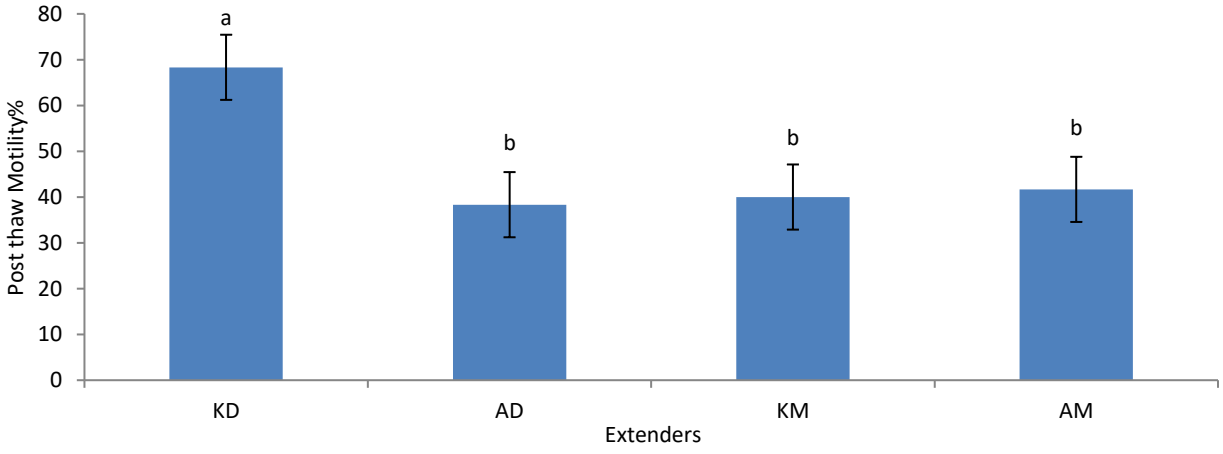

Figure 1. Post thaw motility (mean ± SE) of *Cirrhinus mrigala* sperm cryopreserved in different extenders. Bars marked with the same letter are not significantly different at P<0.05.

DMSO (KD; 68.338±1.67) compared to other extenders such as Kurokura-2 + Methanol (KM; 40±2.89), Alsever's solution + DMSO (AD; 38.33±1.67) and Alsever's solution + Methanol (AM; 41.67±4.40).

Sperm Motility Duration

The effect of different extenders on motility duration (seconds) of *Cirrhinus mrigala* sperm is shown in Figure 2. Sperm motility duration remained maximum in modified Kurokura-1 + DMSO (KD; 78.67±10.41) compared to Kurokura-2 + Methanol (KM; 52.00±5.291), Alsever's solution + DMSO (AD; 46.66±1.66) and Alsever's solution + Methanol (AM; 41.67±1.66).

Sperm Viability

The effect of different extenders on post thaw viability (%) of *Cirrhinus mrigala* sperm is shown in Figure 3. The results showed Kurokura-2 + DMSO (KD) retained significantly ($p<0.05$) higher sperm viability (66.00±1.00) while minimum viability was recorded in Alsever's solution + DMSO (AD; 49.33±5.84). Kurokura-2 + Methanol (KM) and Alsever's solution+ Methanol (AM) maintained percentage post thaw viability of 59.00±3.00 and 55.33±6.38 % respectively, that remained similar to KD extender.

DNA Integrity

The findings of the current study (Figure 4) demonstrate a significant improvement in post-thaw DNA integrity specifically in Kurokura-2 + DMSO (KD), Kurokura-2 + Methanol (KM), and Alsever's solution + Methanol (AM) all exhibited substantially higher post-thaw DNA integrity levels compared to Alsever's solution + DMSO (AD). The graphical representation illustrates post-thaw DNA integrity percentages as follows: 98.67±0.66% for Kurokura-2 + DMSO (KD), 98.00±1.52% for Kurokura-2 + Methanol (KM), 97.33±1.20% for Alsever's solution + Methanol (AM), and 88.00±1.15% for Alsever's solution + DMSO (AD).

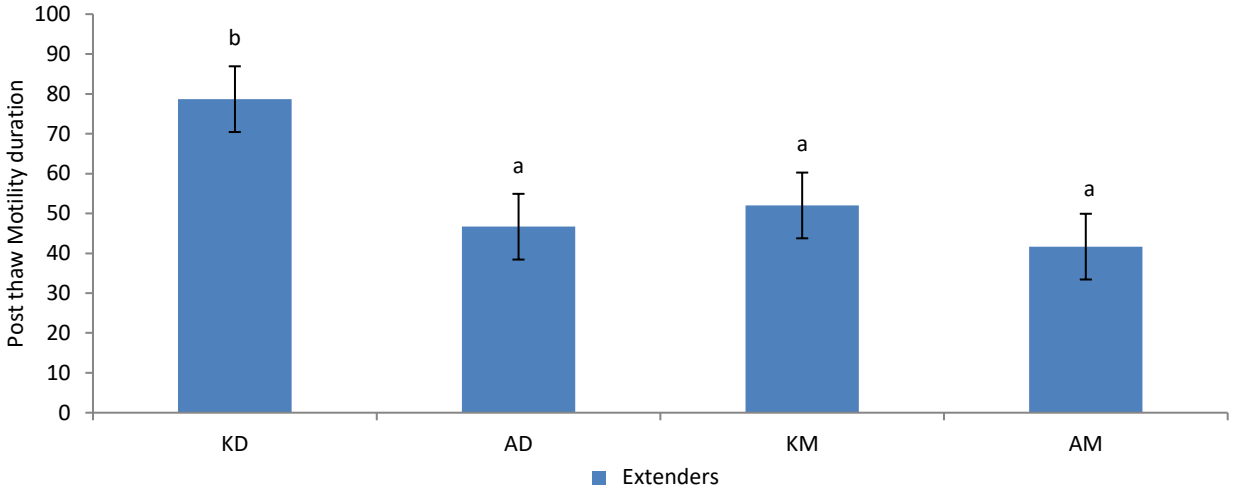

Figure 2. Post thaw motility duration (mean ± SE) of *Cirrhinus mrigala* sperm in different extenders. Bars marked with the same letter are not significantly different at P<0.05.

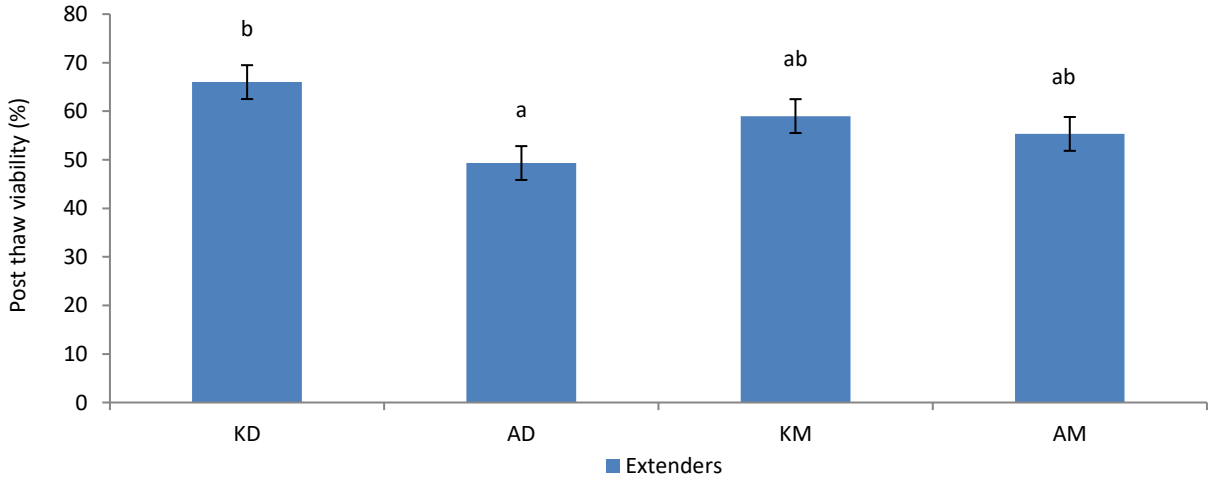

Figure 3. Post thaw viability (mean ± SE) of *Cirrhinus mrigala* sperm in different extenders. Bars marked with the same letter are not significantly different at P<0.05.

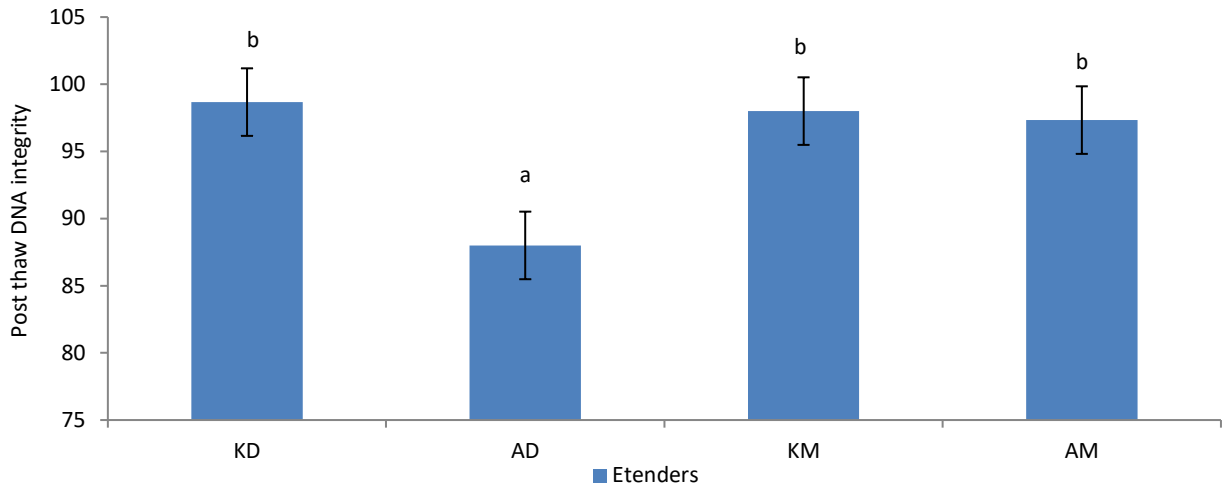

Figure 4. Post thaw DNA integrity (mean ± SE) of *Cirrhinus mrigala* sperm in different extenders. Bars marked with the same letter are not significantly different at P<0.05.

Discussion

Cryopreservation offers a critical solution to challenges faced by carp fish hatcheries, combating issues such as genetic drift, inbreeding, and reduced genetic diversity (Lind et al., 2012). The Food and Agriculture Organization (FAO) recognizes its significance in preserving fish genetic stocks for extended periods. Cryopreservation of fishes has been a topic of interest since the 1950s when first successful experiment on cryopreservation of spermatozoa of *Clupea harengus* (Blaxter, 1953) was established. So far, approximately 200 fish species benefiting from this technique (Blesbois & Labbé, 2003; Hiemstra et al., 2006). However, there's a notable lack of research on sperm cryopreservation in Indian major carps and *Cirrhinus mrigala*.

Milt quality is analyzed in terms of sperm concentration, pH, chemical composition and motilit (Hussain et al., 2018). Motility is an important characteristic for assessing the fresh and cryopreserved milt quality (Lahnsteiner & Patzner, 1998). A study conducted by Verma (Verma, 2009) has reported the sperm motility of *Cirrhinus mrigala* milt in between 74.40±5.17 % and 93.0±3.31%. In the present study, post thaw sperm motility appeared best in Kurokura-1 + DMSO (KD; 68.338±1.67) with motility duration of 78.67±10.41. Motility may be effected due to physical shock that occurs during dilution with extender and cryoprotectant, dilution ratio and equilibration time for penetration of cryoprotectants in spermatozoa, and thawing temperature which is critical for integrity and membrane permeability recovery (Zidni et al., 2020a).

Viability of sperm cells is affected by exposure to different physical and chemical environment. Ice crystal formation during freezing causes high solutes concentration in surrounding matrix that causes swelling and shrinkage of spermatozoa during cryopreservation. Further, osmolarity changes during freezing and thawing also result in membrane damages (Ozkavukcu et al., 2008). In laboratory studies different extenders that behave like the seminal plasma and improve viability of spermatozoa have been reported however, the best medium that mimics the seminal plasma is still to be investigated for different species (Rurangwa et al., 2004), like *Cirrhinus mrigala.* The present study depicted that modified Kurokura extender with 10%DMSO maintained better post thaw viability of *Cirrhinus mrigala* spermatozoa.

The DNA damage occurs due to oxidation of DNA bases resulting in deformation of helical structure of DNA (Zidni et al., 2020b). The responsible elements for this damage are reactive oxygen species (ROS) that exceed the level of antioxidant capacity resulting in greater oxidative stress, the main reason of DNA damage rather than the toxicity of cryoprotectants (Rodrigues et al., 2021). In present study, the Kurokura-2 + DMSO (KD), Kurokura-2 + Methanol (KM), and Alsever's solution + Methanol (AM) extenders were found to maintain DNA integrity of *C. mrigala* sperm compared to Alsever's solution + DMSO (AD) extender. Different species show different effects of cryoinjury owing to specific chromatin structure and the composition of extender along with cryoprotectants (Balamurugan et al., 2019).

The superiority of modified Kurokura extender in cryopreservation of *Cirrhinus mrigala* milt may be attributed to high concentration of potassium, sodium, calcium, magnesium and bicarbonates that serve as buffer during cryopreservation (Chapman, 2016) as well as DMSO is found to be effective cryoprotectant for most fish species (Cabrita et al., 2001) due to its compact molecular structure it easily penetrates and exits the spermatozoa, a crucial attribute for preserving the structural integrity of sperm cell membranes during freezing and thawing procedures (Tiersch et al., 1998). Modified Kurokura extender, due to the qualities of mimicking the seminal plasma of *Cirrhinus mrigala* milt and DMSO as in better compatibility with the extender and *Cirrhinus mrigala* milt provided the best

cryosurvivability and proved to be the best extender for cryopreservation.

The novelty of this research lies in its specific focus on *Cirrhinus mrigala*, a species for which limited research on milt cryopreservation exists. While other studies have explored cryopreservation in various fish species, the unique genetic and physiological characteristics of *Cirrhinus mrigala* necessitate tailored approaches. This study contributes by evaluating and comparing two extenders in combination with DMSO and Methanol, specifically for *Cirrhinus mrigala* milt. The species-specific considerations in extender selection and the varying outcomes in terms of motility, viability, and DNA integrity make this research novel. Additionally, the study addresses the practicality of implementing cryopreservation techniques for genetic conservation and artificial reproduction in *Cirrhinus mrigala*, which can have direct implications for aquaculture and biodiversity management.

Conclusion

In conclusion, the cryopreservation of *Cirrhinus mrigala* milt is a critical technique for maintaining the genetic integrity of this important aquaculture species. This study has evaluated the effectiveness of different extenders for cryopreservation and assessed their impact on post-thaw sperm quality parameters. Among the extenders tested, Kurokura-2 + DMSO (KD) emerged as the most promising, consistently demonstrating superior post-thaw sperm motility, motility duration, viability, and DNA integrity. These findings highlight the potential of KD extender for optimizing the cryopreservation process, ensuring the availability of high-quality milt for selective breeding programs and genetic resource conservation. Further research involving in vivo trials will be valuable to confirm the practical benefits of this cryopreservation protocol. Ultimately, this research contributes to the sustainable management of *Cirrhinus mrigala* populations and enhances the genetic diversity and resilience of this economically significant fish species in aquaculture practices.

Ethical Statement

This study is performed in accordance with the ethical standards set by the Ethical Committee of Pir Mehr Ali Shah Arid Agriculture University Rawalpindi, Pakistan

Funding Information

No funding was received for this article.

Author Contribution

SA: Supervision, study design, review and editing. IA: Investigation, formal analysis, visualization, writing - original draft preparation. SB: Conceptualization, methodology, validation. BAR: Supervision, analysis. SA, MAA, JA: Investigation.

Conflict of Interest

The authors have no conflict of interest to declare.

Acknowledgements

The authors are thankful to administration of Punjab Fish Seed Hatchery, Rawal Town, Islamabad for provision and facilitation in milt collection from *Cirrhinus mrigala* brooders.

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