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Original Article

Effect of L-proline supplementation on long-term cryopreservation of *Piaractus brachypomus* sperm: post-thaw motility, viability, and morphological assessment of sperm

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Abstract
Sperm cryopreservation is widely used in aquaculture for long-term preservation of genetic resources and broodstock management; however, prolonged storage can adversely affect sperm quality due to structural damage and ionic imbalance. This study evaluated the influence of L-proline supplementation, with and without dimethylsulfoxide (DMSO), on the cryopreservation efficiency of *Piaractus brachypomus* sperm. Milt from hormonally induced males was cryopreserved in Modified Cortland Medium (MCM) supplemented with 2, 5, 10, and 30 mM L-proline, either in the absence of DMSO or in combination with 10% DMSO, and maintained under liquid nitrogen for 90 days. Sperm quality after thawing was evaluated at 15-day intervals. Significant differences were observed in sperm motility and viability among treatments. Among the MCM supplemented with L-proline treatments, 10 mM L-proline maintained the highest post-thaw sperm motility (58.05–53.40%) and viability (62.50%), whereas the MCM without cryoprotectant showed the lowest values. Among the MCM containing 10% DMSO treatments, 10% DMSO and 10% DMSO + 2 mM L-proline maintained comparatively higher post-thaw sperm motility (54.36–51.04% and 56.44–49.75%, respectively), whereas other groups exhibited higher post-thaw sperm viability (>52%) than the other DMSO-based treatments. Although a progressive decline in sperm quality was observed over the 90-day storage period, L-proline-supplemented groups maintained superior functional performance. Morphological assessment showed better preservation of sperm head integrity and fewer structural abnormalities in the 10 mM L-proline treatment compared to other cryopreserved treatments. The findings indicate that supplementation with 10 mM L-proline enhances sperm membrane stability and overall cellular function during cryostorage.

Keywords: L-proline; liquid nitrogen storage; membrane stability; modified cortland medium; pacu; sperm cryobiology

1 | INTRODUCTION

Successful seed production in aquaculture depends on the availability of high-quality gametes, particularly sperm exhibiting optimal motility, concentration, and viability (Rurangwa *et al.* 2004). Among the various strategies developed to ensure a consistent supply of viable spermatozoa, cryopreservation has emerged as an important technique widely applied in reproductive management and germplasm preservation for long-term gamete storage and genetic conservation in cultured fish species (Cabrita *et al.* 2010; Martinez-Paramo *et al.* 2017). *Piaractus brachypomus* (Cuvier, 1818), commonly known as Cachama blanca (pirapitinga), is a South American freshwater characiform species naturally distributed in the Amazon basin that performs seasonal reproductive migrations; populations previously attributed to this species in the Orinoco Basin were subsequently demonstrated to represent distinct evolutionary lineages and recognized as separate species following taxonomic revision (Escobar L *et al.* 2015). The species is widely cultivated throughout South America owing to its substantial ecological and commercial importance (Guimaraes and Martins 2015; Jorge *et al*. 2018). The species demonstrates strong, rapid growth and its suitability for culture in diversified farming systems (Kumar *et al.* 2018), contributing to its recognized economic importance in aquaculture across South America (Guimarães and Martins 2015; Jorge *et al.* 2018).

Earlier studies on *P. brachypomus* sperm cryopreservation mainly focused on conventional cryoprotectants such as dimethylsulfoxide (DMSO) and methanol to improve post-thaw sperm quality and fertilization efficiency after thawing (Nascimento *et al.* 2010). Among permeating cryoprotectants, DMSO is commonly used as a cryoprotectant in fish sperm preservation due to its membrane permeability and protective action during freezing and ability to readily permeate biological membranes, promote cellular dehydration, depress the freezing point of intracellular water, and reduce the formation of damaging ice crystals inside sperm cells during the freezing process (Notman *et al.* 2006). However, the use of amino acid-based or naturally occurring cryoprotectants has received limited attention in this species. L-proline, a naturally occurring amino acid, has been reported to enhance cryotolerance by protecting membrane integrity, maintaining osmotic balance, and reducing cellular oxidative damage during freezing and thawing (Abdelnour *et al.* 2024). According to Borges *et al.* (2005) and Gallego and Asturiano (2018), parameters such as motility, sperm concentration, and seminal plasma characteristics are widely used as indicators of sperm functionality and fertilization potential in fish. Optimization of these sperm quality parameters is essential for improving fertilization success and enhancing hatchery efficiency in aquaculture systems (Rurangwa *et al.* 2004; Cabrita *et al.* 2010). Thus, the present study aimed to evaluate the effects of different concentrations of L-proline supplemented in modified Cortland Medium on the cryopreservation of *P. brachypomus* sperm. Post-thaw sperm quality was assessed based on motility, viability, and morphology to identify the optimum L-proline concentration for improving cryopreservation efficiency. The findings may contribute to the development of effective sperm cryopreservation protocols for reproductive management and genetic resource conservation of *P. brachypomus*.

2 | METHODOLOGY

Nine mature adult male *P. brachypomus* brooders (average body weight: 2100±50 g; total length: 48±0.5 cm) were selected for the study during the peak breeding season (November to February) as reported for this species under captive culture conditions (Nascimento *et al.* 2010). The experiment was conducted at the Fisheries College and Research Institute, Thoothukudi, under Dr. J. Jayalalithaa Fisheries University, Tamil Nadu, India.

WOVA-FH (salmon gonadotropin-releasing hormone analogue combined with domperidone; Biostadt India Ltd., India) was administered intramuscularly to mature male brooders at a dose of 0.5 mL kg^{-1} body weight. After a latency period of 10 h, milt was obtained through gentle abdominal stripping using the standard method (Rosengrave *et al.* 2009). All sperm samples were transported to the laboratory at 4°C and processed within 30 min of collection. Precautions were followed to prevent contamination from water, urine, and faecal matter during milt collection (Rurangwa *et al.* 2004; Cabrita *et al.* 2010). Free-flowing milt samples were transferred into pre-labelled sterile containers and maintained at low temperature (4°C) during transport (Asturiano *et al.* 2004). A schematic representation of the cryopreservation procedure adopted in this study is presented in Figure 1.

2.1 Observation of physical properties

Milt volume was estimated directly from the calibrated markings on the collection vials and expressed as mL kg^{-1} brooder's body weight (BW). Only milt samples exhibiting ≥80% initial sperm motility and free from contamination were used for cryopreservation. Visual examination was performed to record milt colour characteristics (Betsy 2013). The pH of milt was determined following the procedure described by Borges *et al.* (2005).

2.2 Analysis of spermatological parameters

2.2.1 Sperm motility: Fresh sperm samples were maintained at approximately (25±2°C) before motility assessment. Sperm motility and motility duration were examined using a light microscope (10× magnification). Motility was assessed immediately after activation (within 10 s) by activating a small aliquot of sperm with distilled water at a dilution ratio of 1:20, following the method described by Ramirez-Merlano *et al.* (2011). Four microscopic fields were examined for each sample, and the average motility value was recorded. A minimum of 200 spermatozoa were counted per slide. All motility assessments were performed by the same observer to ensure consistency throughout the study. Motility score was performed according to the classification system described by Betsy and Kumar (2014). Based on sperm movement, the motility pattern was categorized into forward movement (+++), circular movement (++), and vibratory movement (+) according to the criteria described by Abishag *et al.* (2020).

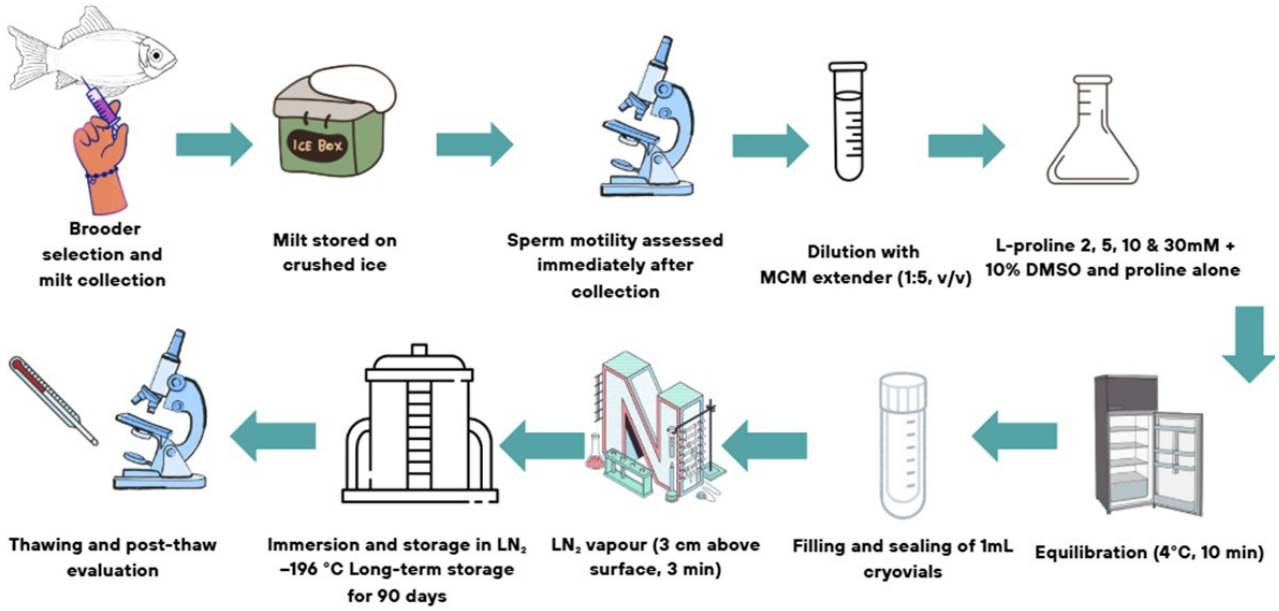

FIGURE 1 Schematic representation of the milt cryopreservation protocol followed in the present study.

2.2.2 Sperm density: Sperm concentration was determined using a Neubauer haemocytometer (NAUBAEUR, Germany). Fresh milt was diluted by mixing 1 µL of sperm with 1200 µL of 0.9% NaCl solution. Sperm concentration was calculated according to the standard Neubauer haemocytometer counting method and expressed as spermatozoa mL^{-1}. Before counting, the loaded hemocytometer chamber was allowed to stabilize under humid conditions for 10 min and then examined at 400X magnification using a light microscope (Medina-Robles *et al.* 2021).

2.2.3 Percentage of live and dead spermatozoa: The sperm viability was assessed using the Eosin–Nigrosin staining technique (Chutia *et al.* 1998), following the procedure described in the carp sperm study (Lollen *et al.* 2024). Grease-free glass slides were coated with 1 µL of diluted milt and 1 µL of eosin-nigrosin stain. The milt with stain was dragged back and forth using a cover slip to ensure proper mixing. The prepared smears were air-dried for 10 to 20 s. A minimum of 200 spermatozoa were counted per slide. Live and dead spermatozoa were differentiated based on eosin–nigrosin stain exclusion characteristics using a binocular microscope (Nikon E360).

2.4 Extender preparation

Modified Cortland Medium (MCM) was used as the sperm extender and prepared according to the formulation described by Truscott *et al.* (1968). The extender consisted of NaCl (1.88 g L^{-1}), KCl (7.20 g L^{-1}), $CaCl_2 \cdot 2H_2O$ (0.23 g L^{-1}), $MgSO_4 \cdot 7H_2O$ (0.23 g L^{-1}), $NaH_2PO_4 \cdot H_2O$ (0.41 g L^{-1}), $NaHCO_3$ (1.00 g L^{-1}), and glucose (1.00 g L^{-1}) (Table 1 and 2). Two sets of extender formulations were prepared: (i) MCM supplemented with L-proline alone (2, 5, 10, and 30 mM; T11, T21, T31, and T41) and (ii) MCM supplemented with 10% (v/v) DMSO in combination with L-proline (2, 5, 10, and 30 mM; T12, T22, T32, and T42). The control treatments consisted of MCM alone (C11) and MCM containing 10% (v/v) DMSO without L-proline (C12). The composition of each extender formulation is presented in Table 1 and 2.

The L-proline concentrations (2, 5, 10, and 30 mM) evaluated in the present study were selected based on previously published studies reporting the cryoprotective effects of L-proline during sperm cryopreservation across various species, including boar (Feng *et al.* 2020), human (Moradi *et al.* 2022), goat (Zhang *et al.* 2022), and buffalo (Ramazani *et al.* 2023). These concentrations were chosen to evaluate the dose-dependent effects of L-proline and to identify the optimum concentration for improving post-thaw sperm quality in *P. brachypomus*. A MCM without DMSO or L-proline was used as the negative control, while an extender containing 10% DMSO without L-proline served as the cryoprotectant control for comparison with the L-proline-supplemented treatments. Although information on the use of L-proline during fish sperm cryopreservation is limited, these concentrations were selected as a rational experimental range to evaluate their dose-dependent effects and to identify the optimum concentration for improving the post-thaw sperm quality of *P. brachypomus*.

Table 1 Chemical composition of Modified Cortland Medium-based extenders supplemented with different concentrations of L-proline for cryopreservation of *Piaractus brachypomus* milt.

Components (per 100 mL)	Experimental groups				
	C11	T11	T21	T31	T41
NaCl (mg)	188	188	188	188	188
KCl (mg)	720	720	720	720	720
$CaCl_2 \cdot 2H_2O$ (mg)	23	23	23	23	23
$MgSO_4 \cdot 7H_2O$ (mg)	23	23	23	23	23
$NaH_2PO_4 \cdot H_2O$ (mg)	41	41	41	41	41
$NaHCO_3$ (mg)	100	100	100	100	100
Glucose (mg)	100	100	100	100	100
L-proline (mM)	0	2	5	10	30

TABLE 2 Chemical composition of Modified Cortland Medium-based extenders supplemented with different concentrations of L-proline and 10% dimethylsulfoxide (DMSO) for cryopreservation of *Piaractus brachypomus* milt.

Components (per 100 mL)	Experimental groups				
	C12	T12	T22	T32	T42
NaCl (mg)	188	188	188	188	188
KCl (mg)	720	720	720	720	720
$CaCl_2 \cdot 2H_2O$ (mg)	23	23	23	23	23
$MgSO_4 \cdot 7H_2O$ (mg)	23	23	23	23	23
$NaH_2PO_4 \cdot H_2O$ (mg)	41	41	41	41	41
$NaHCO_3$ (mg)	100	100	100	100	100
Glucose (mg)	100	100	100	100	100
DMSO (% v/v)	10	10	10	10	10
L-Proline (mM)	0	2	5	10	30

Fresh milt samples were diluted with each prepared extender at a ratio of 1:5 (v/v), following the cryopreservation protocol described by Ramirez-Merlano *et al.* (2011). The diluted samples were equilibrated at 4°C for 10 min to facilitate cryoprotectant permeation and stabilize the sperm plasma membrane before freezing. Following equilibration, the samples were loaded into sterile 1 mL cryovials and tightly sealed. The cryovials were suspended 3 cm above the surface of liquid nitrogen for 3 min to achieve controlled vapour freezing and were subsequently immersed in liquid nitrogen (–196°C) and stored in a BA11 cryocan (IBP, India). Cryopreserved samples were stored for 90 days, and post-thaw sperm quality assessments were performed at 15-day intervals (15, 30, 45, 60, 75, and 90 days).

2.5 Post-thaw analysis

Cryovials were thawed individually in a thermostatically controlled water bath at 37°C for 60 s. Immediately after thawing, sperm quality was evaluated to minimize post-thaw deterioration. Post-thaw analysis included sperm motility, viability, and morphological characteristics using the methods described in the respective subsections. All analyses were performed under identical laboratory conditions for each treatment and storage period.

2.6 Scanning Electron Microscopy

The ultrastructural characteristics of spermatozoa were observed through scanning electron microscopy (SEM). Sperm samples were preserved overnight at 4°C using 2.5% glutaraldehyde prepared in 0.1 M sodium phosphate buffer (pH 7.2). Following fixation, samples were washed and dehydration was carried out sequentially using increasing concentrations of ethanol solution (50%, 70%, 90%, and 100%) for 15 min each. Mixtures of 100% ethanol and hexamethyldisilazane (HMDS) at ratios of 3:1, 1:1, and 1:3 were used for chemical drying for 15 minutes each. After that, the mixture was treated with 100% HMDS and allowed to air dry overnight.

The ICAR-Central Institute of Brackishwater Aquaculture (CIBA), Chennai, India, used a scanning electron microscope (9JSM-IT300LV, JEOL Ltd., Tokyo, Japan) to mount and examine the dried samples. A secondary electron detector (SED) with a working distance of 9.3 mm was used for imaging under high vacuum conditions at an accelerating voltage of 15 kV. Micrographs were captured at magnifications ranging from x4,300 to x7,000 with an integrated scale bar. Morphological abnormalities were assessed microscopically by examining 200 spermatozoa per treatment. The percentages of normal spermatozoa were calculated from the total number of sperm cells examined.

2.7 Experimental design

The experiment comprised two treatment groups to eval-

uate the effect of L-proline supplementation on the cryopreservation of *P. brachypomus* sperm: (i) MCM-based extenders supplemented with different concentrations of L-proline (0, 2, 5, 10, and 30 mM) without DMSO and (ii) MCM-based extenders supplemented with the same concentrations of L-proline in the presence of 10% (v/v) DMSO. The experiment was conducted in triplicate. For each replication, milt was collected from nine mature male brooders, pooled to minimize individual variation, and aliquoted for dilution with the respective experimental extenders.

2.8 Data analysis

All experimental data are presented as mean ± standard deviation (SD). Statistical analyses were performed using SPSS software (version 20.0; IBM Corp., Armonk, NY, USA). The data were tested for normality using the Shapiro–Wilk test and for homogeneity of variances using Levene's test prior to statistical analysis. Sperm motility (%) and viability data were analyzed using two-way analysis of variance (ANOVA) to evaluate the effects of treatment, storage period, and their interaction. When a significant interaction was detected, simple main effects were further analyzed using one-way ANOVA within each storage period, followed by Tukey's honestly significant difference (HSD) post hoc test for multiple comparisons. Sperm morphology data were analyzed using one-way ANOVA followed by Tukey's HSD post hoc test. Differences were considered statistically significant at $p < 0.05$.

3 | RESULTS

3.1 Fresh milt characteristics

The fresh milt characteristics of *P. brachypomus* are presented in Table 3. The average milt volume was 8.00 mL and milky white. Milt pH was recorded as 8.00±0.10. Sperm motility was 87.00±2.00% with a motility duration of 50±2 s. Motility pattern was observed as +++, with a motility score of 10.00. Sperm viability was 92.00±2.00%, and sperm density was estimated as (1.80±0.17)×10^{10} spermatozoa mL^{-1} (Table 3).

TABLE 3 Fresh milt characteristics of *Piaractus brachypomus* (n = 9 brooders; values expressed as mean ± SD).

Parameter	Value (Mean ± SD)
Milt volume (ml)	8.00
Colour	Milky white
Milt pH	8.00±0.1
Motility (%)	87.00±2.00
Duration (s)	50±2
Motility pattern	+++
Motility score	10.00
Sperm viability	92.00±2.00
Sperm density (spermatozoa mL^{-1})	(1.80±0.17)×10^{10}

3.2 Post-thaw sperm quality parameters

3.2.1 Sperm motility (%): Post-thaw sperm motility was highest in the 10 mM L-proline treatment (T31), which was significantly higher ($p < 0.05$) than the other treatments in the MCM supplemented with L-proline group. In the MCM containing 10% DMSO supplemented with L-proline group, C12 and T12 exhibited significantly higher sperm motility than the remaining treatments ($p < 0.05$). Sperm motility declined with increasing storage duration. Two-way ANOVA revealed significant effects of treatment, storage duration, and their interaction ($p < 0.001$) (Tables 4 and 5).

TABLE 4 Effect of L-proline supplementation in Modified Cortland Medium (MCM) on post-thaw sperm motility (%) during cryostorage.

Treatments	Days					
	15 d	30 d	45 d	60 d	75 d	90 d
C11 (MCM without cryoprotectant)	26.49±2.08^{e}	26.03±0.82^{e}	25.03±0.82^{d}	22.85±2.04^{d}	21.70±1.95^{c}	21.54±1.24^{d}
T11 (2 mM L-proline)	41.70±1.24^{d}	40.70±1.24^{d}	40.02±0.82^{c}	40.37±1.26^{c}	40.37±1.26^{b}	40.05±1.44^{c}
T21 (5 mM L-proline)	50.36±1.26^{bc}	50.34±0.47^{bc}	49.01±0.82^{b}	48.68±0.95^{b}	48.01±0.82^{a}	48.00± 0.47^{b}
T31 (10 mM L-proline)	58.05±1.64^{a}	58.36±1.26^{a}	55.45±2.48^{a}	53.39±1.69^{a}	54.15±2.89^{a}	53.40±1.91^{a}
T41 (30 mM L-proline)	43.02±0.82^{d}	41.70±1.24^{d}	39.67±0.47^{c}	39.34±0.82^{c}	39.04±0.48^{b}	38.71±1.24^{c}

Values are expressed as mean ± SD (n = 3). Different superscript letters within the same column indicate significant differences among treatments at each storage day ($p < 0.05$; one-way ANOVA followed by Tukey's HSD test).

3.2.2 Sperm viability: After cryopreservation, the highest sperm viability was observed in the 10 mM L-proline treatment (T31), which was significantly higher ($p < 0.05$) than the other treatments in the MCM supplemented with L-proline group. In the MCM containing 10% DMSO supplemented with L-proline group, C12 and T22 exhibited significantly higher sperm viability than the remaining treatments ($p < 0.05$). Sperm viability gradually declined from day 15 to day 90, with no significant difference between days 75 and 90. No significant interaction was ob-

served between treatment and storage duration (p > 0.05) (Figures 2 and 3).

3.2.3 Sperm morphology: After 45 days of cryopreservation, sperm morphology was assessed in four selected treatments (T12, T31, C11, and C12) representing two cryoprotectant groups. Sperm morphology differed significantly among treatments (p < 0.05). Among the MCM supplemented with L-proline treatments, the 10 mM L-proline concentration (T31) showed the highest percentage of normal spermatozoa. Among the MCM containing 10% DMSO treatments, 10 % DMSO + 2mM L- proline (T12) showed the highest percentage of normal spermatozoa (Figures 4 and 5).

TABLE 5 Effect of 10% dimethylsulfoxide (DMSO) and L-proline supplementation in Modified Cortland Medium (MCM) on post-thaw sperm motility (%) during cryostorage.

Treatment	Days					
	15 d	30 d	45 d	60 d	75 d	90 d
C12 (10% DMSO)	54.36±1.26^{ab}	54.01±0.82^{ab}	51.70±1.25^{ab}	50.36±1.26^{ab}	51.04±1.43^{a}	51.04±1.43^{ab}
T12 (10% DMSO + 2 mM L-proline)	56.44±2.48^{ab}	55.74±2.05^{ab}	51.70±1.25^{ab}	51.36±1.26^{ab}	50.00±0.00^{a}	49.75±0.43^{b}
T22 (10% DMSO + 5 mM L-proline)	44.81±2.53^{cd}	44.32±3.80^{cd}	43.60±3.47^{c}	41.37±1.26^{c}	40.34±0.48^{b}	40.00±0.00^{c}
T32 (10% DMSO + 10 mM L-proline)	42.40±1.69^{d}	42.05±1.40^{d}	41.35±0.94^{c}	40.34±0.48^{c}	39.02±0.82^{b}	38.34±0.48^{c}
T42 (10% DMSO + 30 mM L-proline)	40.70±1.24^{d}	39.81±2.41^{d}	39.34±0.48^{c}	38.71±1.24^{c}	37.38±1.26^{b}	36.67±0.47^{c}

Values are expressed as mean ± SD (n = 3). Different superscript letters within the same column indicate significant differences among treatments at each storage day (p < 0.05; one-way ANOVA followed by Tukey's HSD test).

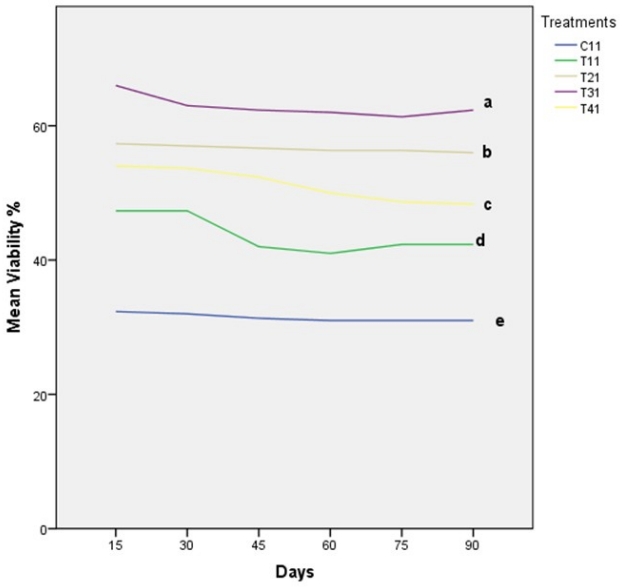

FIGURE 2 Changes in sperm viability (%) during cryostorage using Modified Cortland Medium (MCM) supplemented with different concentrations of L-proline. Values are expressed as mean ± SD (n = 3). Different lowercase letters indicate significant differences among treatment means according to Tukey's HSD test (p < 0.05).

4 | DISCUSSION

This work is the first thorough report on the use of L-proline as a cryoprotectant in fish milt for sperm cryopreservation. The findings demonstrate that *P. brachypomus* spermatozoa can be effectively cryopreserved using various concentrations of L-proline either alone or in combination with DMSO. Although maintaining the superior quality of live fish is inherently challenging, high-quality germplasm can be efficiently preserved through cryopreservation techniques. The present study further indicates that sperm quality can be maintained over extended storage periods. Notably, the use of L-proline as a cryoprotectant is a novel approach not extensively explored in prior studies.

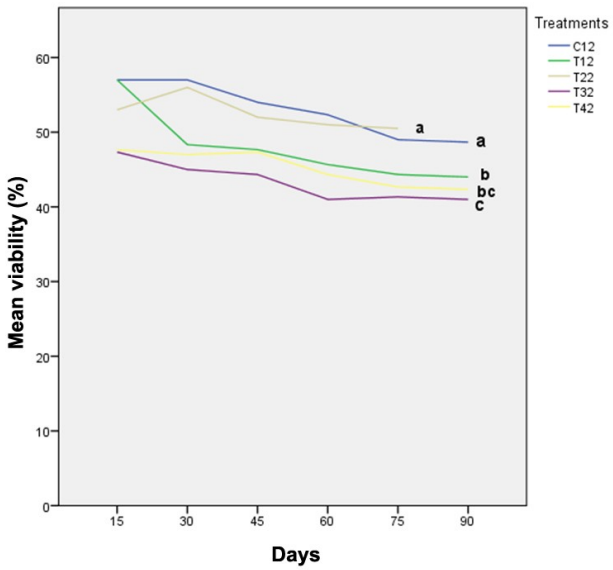

FIGURE 3 Changes in sperm viability (%) during cryostorage using Modified Cortland Medium (MCM) containing 10% DMSO supplemented with different concentrations of L-proline. Values are expressed as mean ± SD (n = 3). Different lowercase letters indicate significant differences among treatment means according to Tukey's HSD test (p < 0.05).

The present study demonstrated that fresh sperm of *P. brachypomus* showed a highest motility rate of 87.00±2.00%. This observation is consistent with earlier findings reported for the same species by Fresneda *et al.* (2004) and Navarro *et al.* (2004), who documented motility values close to 86%. Comparable sperm motility levels have also been observed in other characid fishes, including *Brycon amazonicus* (Cruz-Casallas *et al.* 2006). The motility duration was recorded as 50±2 s, which is lower than the longer motility duration (100.6 s) reported in *P. brachypomus* by Fresneda *et al.* (2004).

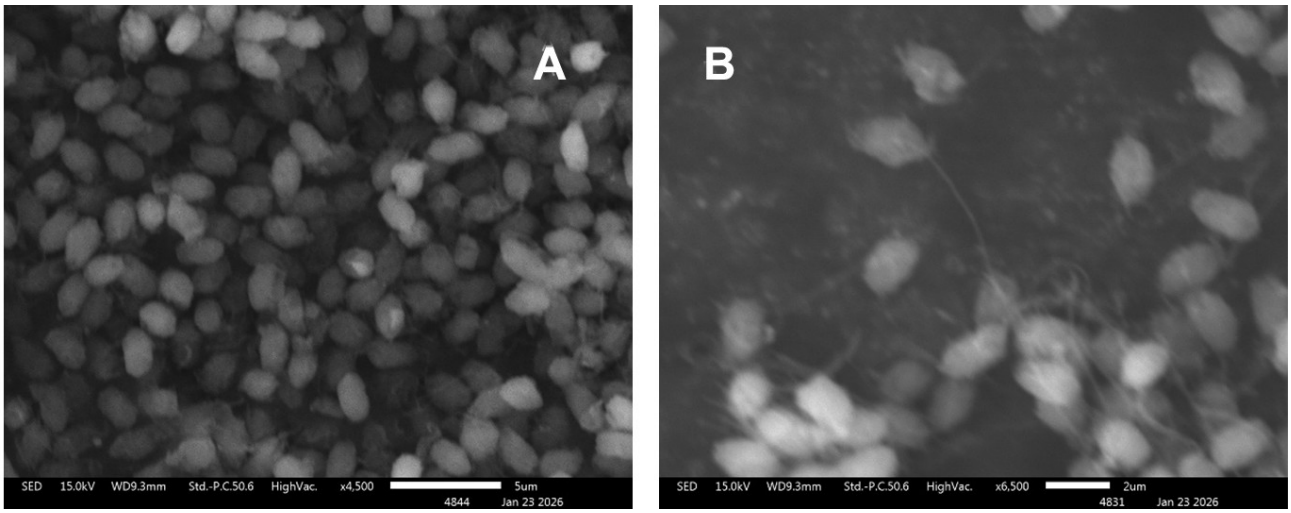

FIGURE 4 Scanning electron micrographs (SEM) of cryopreserved spermatozoa in Modified Cortland Medium (MCM) supplemented with L-proline. (A) C11 (MCM) (MCM alone) showing altered sperm head morphology and surface irregularities. (B) Treatment 31 (10mM L-proline) showing well-preserved sperm head structure with smooth surface.

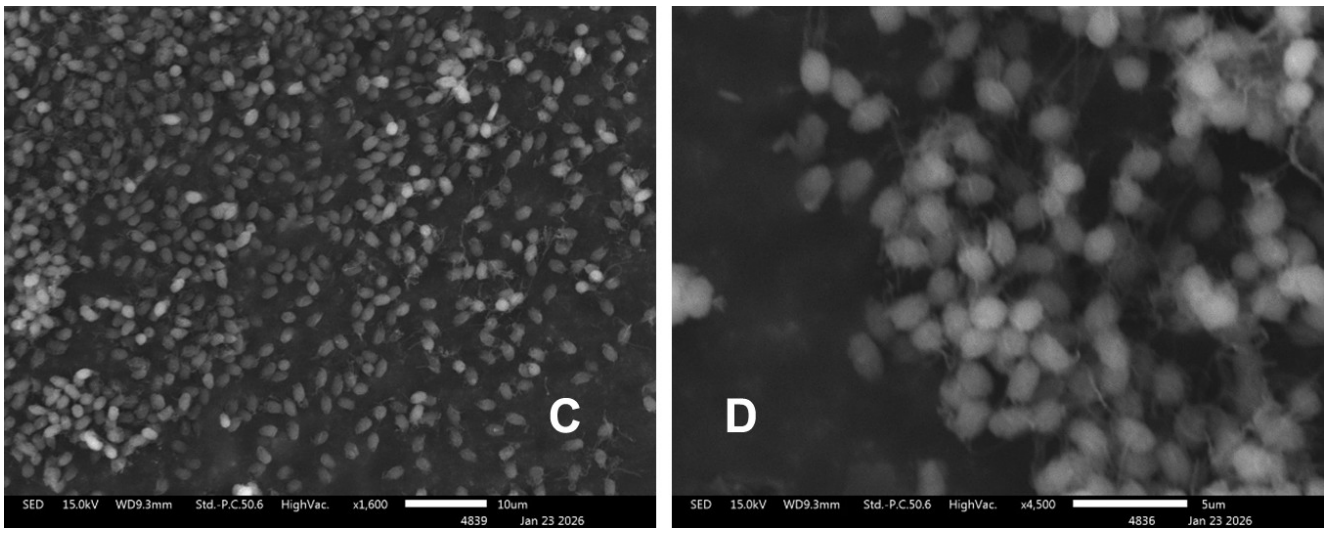

FIGURE 5 Scanning electron micrographs (SEM) of cryopreserved spermatozoa in Modified Cortland Medium (MCM) containing 10% DMSO and supplemented with L-proline. (C) C12 (10% DMSO) showing distorted and elongated sperm heads. (D) Treatment 12 (10 % DMSO + 2mM L-proline) showing intact and uniform sperm head morphology

The average seminal volume obtained from brooders was 8.00 mL, and this value appeared relatively greater than those described for other South American Characidae species, including *P. lineatus*, which showed a seminal volume of 2.2 mL (Viveiros and Godinho 2009). The fresh milt of *P. brachypomus* exhibited a sperm concentration of (1.80±0.17)×10^{10} spermatozoa mL^{-1}. This concentration was comparatively lower than the value reported by Nascimento *et al.* (2010) for the same species, where an average sperm density of about 5.55×10^{10} spermatozoa mL^{-1} was recorded. In contrast, the sperm concentration observed in the present study was greater

than the values previously described for *B. orbignyanus*, including 5.7±3.4 $\times 10^9$ spermatozoa mL^{-1} reported by Di Chiacchio *et al.* (2017) and 30.6$\times 10^9$ spermatozoa mL^{-1} reported by Fresneda *et al.* (2004). In the present investigation, the sperm viability of *P. brachypomus* was determined to be 92.00±2.00%, reflecting good membrane stability and overall semen quality. The viability percentage obtained was marginally lower than the 95% reported by Navarro *et al.* (2004), but exceeded the 86% value documented by Fresneda *et al.* (2004) for the same species. Differences in sperm viability reported across studies could be associated with factors such as broodstock health, semen handling techniques, environmental conditions, and stress experienced during gamete collection (Cabrita *et al.* 2010). Nevertheless, the semen characteristics observed in this study were within the normal range described for characiform fishes, indicating that the induced breeding protocol successfully produced healthy and viable sperm appropriate for subsequent cryopreservation studies.

Cryopreservation of fish sperm commonly employs nitrogen vapour as the primary freezing method (Cruz-Casallas *et al.* 2006). Throughout the freezing and thawing process, sperm cells undergo several physical and biochemical alterations, including formation of ice crystals, mechanical injury, and damage to the plasma membrane structure (Lahnsteiner *et al.* 1992; Labbe *et al.* 1997). In the present study, 1 mL cryovials (Ott and Horton 1971) were used, which may have facilitated efficient thermal transfer during both freezing and thawing at 37°C for 60 s, thereby contributing to improved post-thaw sperm quality.

Post-thaw sperm motility is widely recognized as a key indicator of cryopreservation efficiency because it reflects the functional integrity and fertilizing potential of sperm following thawing (Cabrita *et al.* 2010; Viveiros *et al.* 2012). In the present study, among the MCM supplemented with L-proline treatments, 10 mM L-proline (T31) consistently maintained the highest post-thaw sperm motility (58.05–53.40%) throughout the storage period. Similarly, among the MCM containing 10% DMSO treatments, 10% DMSO + 2 mM L-proline (T12) exhibited comparatively higher post-thaw sperm motility (56.44–49.75%), followed by 10% DMSO alone (C12) (54.36–51.04%), indicating that L-proline supplementation enhanced sperm cryosurvival under both extender systems. Kutluyer *et al.* (2016) reported the highest post-thaw sperm motility (72.50 ± 3.54%) in *Carassius auratus* at 4 mM taurine, which was higher than the values observed in the present study. In *P. brachypomus*, Navarro *et al.* (2004) recorded post-thaw motility rates of 40% with 10% DMSO, 37% with 5% ethylene glycol, and 57% with 10% methanol. The maximum motility obtained with 10 mM L-proline in the present study (58.05%) was slightly higher than the value reported with methanol by Navarro *et al.* (2004). Likewise, Nascimento *et al.* (2010) reported a lower subjective post-thaw motility (49±22%) using DMSO in the same species. Higher motility values (80% with DMSO and 78% with methanol) were reported by Fresneda *et al.* (2004), whereas Cabrita *et al.* (2001a and 2001b) observed a comparatively lower post-thaw motility (45%) in *Oncorhynchus mykiss* using 7% DMSO. The variation in post-thaw sperm motility among studies may be attributed to differences in species, cryoprotectant type and concentration, extender composition, freezing and thawing protocols, packaging volume, and species-specific membrane characteristics. The superior motility observed with L-proline supplementation, particularly at 10 mM in MCM and 2 mM in combination with 10% DMSO, may be associated with its osmoprotective and membrane-stabilizing properties, which help minimize cryoinjury during freezing and thawing. Furthermore, De Baulny *et al.* (1997) reported that effective cryopreservation preserves sperm membrane integrity and mitochondrial function, supporting the improved post-thaw motility observed in the present study.

Sperm viability is widely recognized as an important indicator of cryopreservation efficiency because it represents the integrity of the sperm membrane and the ability of spermatozoa to successfully fertilize eggs (Cabrita *et al.* 2001; Li *et al.* 2010). In the present study, fresh semen exhibited a viability of 92±2.00% before cryopreservation. Following freezing and thawing, viability declined in all treatments to nearly 30%; however, the highest viability was recorded in the treatment containing 10 mM L-proline (T31), which showed 60.33±0.58% viability at 90 days of storage. Moreover, the lack of significant variation between 75 and 90 days indicates that sperm quality and membrane stability were successfully preserved during the later stages of cryostorage. The values obtained in the current study were greater than those previously reported for *P. brachypomus* by Navarro *et al.* (2004), who recorded values ranging from 0 to 29.4%. Variations among studies may arise from multiple factors such as differences in cryoprotectant toxicity among species (Santana *et al.* 2020), freezing conditions (Balamurugan and Munuswamy 2017), membrane lipid composition (Bai *et al.* 2019), semen dilution ratio (Marco-Jiménez *et al.* 2006), and the combined influence of these factors during the cryopreservation process (Gaitan-Espitia *et al.* 2013).

The present study revealed that a significantly lower percentage of abnormal spermatozoa (34.0±2.6%) was observed in the 10 mM L-proline concentration (T31) in the MCM supplemented with L-proline group. These findings clearly indicate that L-proline supplementation markedly reduced cryo-induced structural damage during freezing and thawing. Lower sperm abnormality percentages have been documented in earlier studies, including the findings of Medina-Robles *et al.* (2023), who reported abnormalities ranging from 12.1 to 14.3% after extended

cryostorage, as well as in common carp, where Linhares *et al.* (2015) recorded 19.4% abnormalities. The relatively elevated abnormality levels observed in the current study could be associated with variations among species in membrane lipid composition, differences in cryoprotectant composition, freezing conditions, duration of storage, and the methods used for sperm evaluation. The percentage of sperm abnormalities observed in the 10 mM L-proline treatment was comparatively lower than the values previously reported for several teleost fishes. Higher abnormality rates have been documented in Amazon catfish (*Leiarius marmoratus*) (64.50±12.46%) (Borges *et al.* 2020), piracanjuba (*Brycon orbignyanus*) (45.60±4.15%) (Galo *et al.* 2011), pejerrey (*Odontesthes bonariensis*) (60%) (Garriz and Miranda 2013), and tambaqui (61.3±8.1%) (Medina-Robles *et al.* 2019). These findings indicate that the inclusion of L-proline in the extender may have contributed to better maintenance of sperm structural integrity in the present study when compared with several other tropical freshwater fish species. Variations observed among different studies may be influenced by species-specific responses to cryopreservation, differences in the protective efficiency of cryomedia, and reproductive seasonal conditions.

5 | CONCLUSIONS

The present study demonstrated that supplementation with 10 mM L-proline significantly enhanced the post-thaw quality of fish sperm, indicating its potential as an effective additive in cryopreservation protocols. L-Proline, which has been widely studied in mammalian systems such as buffalo, ram, goat, donkey, and human, has shown promising applicability in fish as well, marking it as a novel approach in aquaculture research. The present findings indicate that L-proline may improve sperm motility, viability, and cellular integrity. Unlike many conventional cryoprotectants, L-proline makes it a safer alternative for long-term germplasm preservation. The findings of the present study indicate that L-proline can serve as an effective, environmentally friendly, and biologically compatible cryoprotectant for the cryopreservation of fish sperm. Additional investigations are needed to determine the optimal L-proline concentration and evaluate its effectiveness across a wider range of fish species.

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ETHICAL APPROVAL

The study protocol was approved by the Ethics Committee of the Tamil Nadu Dr. J. Jayalalithaa Fisheries University, Nagapattinam (TNJFU), India.

CONFLICT OF INTEREST

The author declares no conflict of interest.

AUTHORS' CONTRIBUTION

Author Contribution Declaration: S. Mariammal: Writing – original draft, Methodology, Data curation. J. Jaculine Pereira: Conceptualization, Review & Editing, Supervision, Investigation. R. Somu Sunder Lingam: Supervision, Investigation. A. Anix Vivek Santhiya: Supervision, Investigation. R. Shalini: Supervision, Investigation.

AI-USE STATEMENT

During the preparation of this work, the author used ChatGPT (Open AI) for grammar correction and language editing. After using this tool, the author (s) reviewed and edited the content as needed and take full responsibility for the content of the publication.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on a reasonable request from the corresponding author.

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