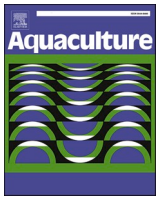
Aquaculture 598 (2025) 741985

Contents lists available at ScienceDirect

Aquaculture

journal homepage: www.elsevier.com/locate/aquaculture



Regulatory role of exosomal proteins MIF and PEBP1 in sperm storage in black rockfish (*Sebastes schlegelii*) ovary

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A R T I C L E I N F O

Keywords:
Black rockfish
Exosomes
MIF
PEBP1
Female sperm storage

A B S T R A C T

Sperm entry into the female reproductive tract triggers the production of exosomes that play a vital role in regulating sperm storage, release, and viability. Exosomal proteins are considered to be key factors influencing these processes. In this study, we focused on macrophage migration inhibitory factor (MIF) and phosphatidylethanolamine-binding protein 1 (PEBP1) to investigate their amino acid sequence identification, expression patterns, and functional roles in post-mating ovarian tissues of black rockfish (*Sebastes schlegelii*). Phylogenetic analysis revealed that MIF and PEBP1 cluster separately across species, with distinct groupings for fish and mammals, underscoring their evolutionary conservation and essential biological roles. We observed significant upregulation of *mif* and *pebp1* gene expression during various stages of post-mating sperm storage, which was reflected in corresponding increases in protein levels. Exosomes were successfully isolated from the ovarian fluid of black rockfish, with particle sizes ranging from 40 to 220 nm, and exhibiting typical exosomal morphological characteristics. More importantly, the presence of MIF and PEBP1 was confirmed in these exosomes. Both proteins were localized in ovarian follicular and epithelial cells. Functional analysis revealed that recombinant *Sebastes schlegelii* MIF (rSsMIF) and PEBP1 (rSsPEBP1) were capable of transferring into sperm after incubation, suggesting their binding interaction. Both proteins effectively preserved sperm integrity, including intact heads and undamaged flagella. After 24 h of incubation with rSsMIF and rSsPEBP1, sperm viability was maintained at 88 % and 85 %, respectively, compared to only 58 % in the control group. Additionally, sperm motility was also significantly improved following treatment with both proteins, with the proportion of motile sperm increasing by 14 % and 20 %, respectively. Moreover, the present study found that the regulation of sperm viability and motility by two types of exosomal proteins may accompanied by intracellular reactive oxygen species (ROS) and calcium ions. The levels of ROS in sperm decreased to varying extents after incubation with the two proteins, whereas intracellular calcium ion concentrations increased. These findings provide new insights into the roles of MIF and PEBP1 in sperm storage and fertility, suggesting potential applications in reproductive biotechnology.

1. Introduction

The sperm storage mechanism is crucial for allowing sperm to temporarily reside in the female reproductive tract and be released at the optimal time after ovulation, ensuring successful fertilization (Burns et al., 1995; Orr and Brennan, 2015; Qazi et al., 2003). Fish, being the most diverse group of vertebrates, exhibit various reproductive modes including oviparity, ovoviviparity and viviparity. In ovoviviparous and viviparous fish, fertilization predominantly occurs internally (Jalabert, 2005; Teletchea et al., 2009). In black rockfish, a viviparous teleost, sperm are stored in the ovaries for approximately five months, occupying different positions during various storage periods (He et al., 2019). Using integrative multi-omics approaches, the female sperm storage process in black rockfish was previously investigated (Li et al., 2024b).

Exosomes are small membrane-bound vesicles containing a complex of RNA and proteins, with a specific reference to disc-shaped vesicles with diameters ranging from 40 to 200 nm (Kalluri and LeBleu, 2020; Théry et al., 2002). It was found recently that exosomes are widely present in semen (Vojtech et al., 2014), epididymal fluid (Baskaran

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https://doi.org/10.1016/j.aquaculture.2024.741985
Received 28 September 2024; Received in revised form 15 November 2024; Accepted 30 November 2024
Available online 3 December 2024

et al., 2020), endometrial fluid (Ng et al., 2013), and follicular fluid (Santonocito et al., 2014), where they transport biologically active cargo such as proteins, lipids, and nucleic acids. They have been shown to influence processes in both the female and male reproductive systems, including gametogenesis, acrosome reaction, sperm capacitation, and embryo implantation in the endometrium (Greening et al., 2016; Machtinger et al., 2016; Sullivan et al., 2005). Seminal exosomes, membranous vesicles present in seminal plasma, are crucial components of the seminal plasma microenvironment; they are taken up by sperm through membrane fusion mechanisms and play roles in the maturation and functional regulation of sperm (Du et al., 2016; Murdica et al., 2019). While the roles of seminal exosomes in the regulation of sperm maturation and motility are well-documented, there are still limited reports on the regulatory effects of uterine or ovarian fluid exosomes on sperm within the female reproductive tract. Our recent research has confirmed the role of ovarian fluid exosomes in maintaining sperm motility in black rockfish (Chen et al., 2024), but the key proteins involved need further investigation.

Exosomal proteins play a crucial regulatory role in recipient cells, making them a focal point of exosome research (Li et al., 2017; Li et al., 2023). In this study, we identified macrophage migration inhibitory factor (MIF) in the ovarian fluid exosomes of black rockfish, which piqued our interest. MIF was initially described as a product that activates T lymphocytes and inhibits the random migration of cultured macrophages. However, it is now recognized as playing a broader role in various biological processes, including inflammation and immune responses (Jankauskas et al., 2019; Roger et al., 2001). Notably, some studies have reported a negative correlation between the levels of sperm-associated MIF and sperm motility in different semen samples (Carli et al., 2007). MIF is abundantly expressed in the epithelial cells of the rat epididymis and in the outer dense fibers of epididymal sperm, where it is transferred to sperm cells via epididymosomes (Eickhoff et al., 2001). Additionally, CD9-positive vesicles in bovine cauda epididymal fluid, which are a subpopulation of epididymosomes, contain proteins involved in sperm maturation, such as P25b, GliPr1L1, and MIF (Caballero et al., 2013). While the role of MIF in male reproduction, particularly in spermatogenesis and sperm maturation, has been extensively studied, its function in female reproduction remains poorly understood, with limited studies exploring its potential involvement.

Another exosomal protein, phosphatidylethanolamine-binding protein 1 (PEBP1), a member of the phosphatidylethanolamine-binding protein family, was also identified in the ovarian fluid exosomes of black rockfish. This protein regulates various signaling pathways, including MAPK, NF-kappa B, and glycogen synthase kinase 3 (GSK-3) signaling pathways (Rajkumar et al., 2016), and may be involved in neural development. Additionally, PEBP1 is associated with many human cancers and may act as a metastasis suppressor gene (Ahmed et al., 2021). Remarkably, like MIF, PEBP1 was also identified as a protein in bovine epididymosomes (Frenette et al., 2010; Girouard et al., 2011). Furthermore, PEBP1 exhibits significant differential expression profiles in the sperm and plasma proteomes of high-fertility and low-fertility Holstein bulls (Kasimanickam et al., 2019).

This study focuses on the viviparous black rockfish, a species capable of long-term sperm storage within the ovary, to elucidate the potential roles of ovarian fluid exosomal proteins MIF and PEBP1 in this process. The results revealed that the expression levels of MIF and PEBP1 were significantly upregulated in ovarian tissues post-mating, and both proteins were identified in exosomes isolated from ovarian fluid after copulation. More importantly, we confirmed that MIF and PEBP1 can be transferred to sperm, modulating intracellular ROS levels and calcium ion content, ultimately influencing sperm morphological integrity, survival rate, and motility. In conclusion, MIF and PEBP1 may function as extracellular secretory proteins within the ovarian environment, acting on stored sperm to promote their long-term stability.

2. Materials and methods

2.1. Ethics statement

All animal experiments were conducted according to the guidelines for the care and use of animals for scientific purposes and were carried out following the protocols of the Institutional Animal Care and Use Committee of the Ocean University of China and the China Government Principles for the Utilization and Care of Vertebrate Animals Used in Testing, Research, and Training.

2.2. Fish and samples

This study utilized 30 healthy male and 30 female (3-year-old) black rockfish for rearing and sampling. The fish were sourced from PuWan quay market (Qingdao, Shandong, China). The rearing conditions, including water temperature, salinity, and net cage dimensions, were referenced from previous study (Wang et al., 2023b). Initially, the males and females were kept separately to prevent natural mating. Subsequently, to obtain specific and accurate sperm storage durations, we performed artificial insemination experiments based on previous study (Li et al., 2024b), resulting in 15 inseminated and 15 non-inseminated female groups. According to the duration of sperm storage and previous study (Li et al., 2024b), the stages were classified into early sperm storage stage (ESS), mid-term sperm storage stage (MSS), and late sperm storage stage (LSS), with at least six inseminated and three non-inseminated females sampled at each stage. After anesthesia, one-half of the ovarian tissue was removed for RNA extraction and Immunohistochemistry, and the other half was used for the exosomal extraction.

2.3. Identification of SsMIF or SsPEBP1 cDNA and bioinformatics assay

The amino acid sequences of *Sebastes schlegelii* MIF (SsMIF, *Ssc.10018577*) and *Sebastes schlegelii* PEBP1 (SsPEBP1, *Ssc. 10023540*) were obtained from CNSA (CNGB Nucleotide Sequence Archive) under the accession ID CNP0000222. ORF, amino acid sequence, and molecular weight analyses were conducted using DNASTAR. A phylogenetic tree of MIF and PEBP1 from 11 species (*Homo sapiens*, *Mus musculus*, *Gallus gallus*, *Xenopus tropicalis*, *Lepisosteus oculatus*, *Danio rerio*, *Takifugu rubripes*, *Oreochromis niloticus*, *Oryzias latipes*, *Sebastes umbrosus*, and *Sebastes schlegelii*) was constructed using the Neighbor-Joining method in MEGA. The nucleotide and amino acid sequences of other species were obtained from NCBI and are detailed (Supplementary Table 1).

2.4. Expression profiling of SsMIF and SsPEBP1 based on qRT-PCR and proteome datasets

The mRNA expression profiles of the *mif* and *pebp1* in the ovaries of black rockfish at three different sperm storage stages were analyzed using qRT-PCR. RNA extraction, cDNA synthesis, and qRT-PCR were performed following established protocols (Wang et al., 2023a). The qRT-PCR was conducted using a LightCycler 480 system (Roche, Forrentrasse, Switzerland) with a total reaction volume of 20 μL, containing 10 ng of template cDNA, 0.4 μL of each primer, and 10 μL of NovoStart®SYBR qPCR SuperMix plus (Novoprotein, Shanghai, China). Each sample was run in triplicate. The relative mRNA expression levels were quantified using the comparative Ct method ($2^{-\Delta\Delta Ct}$) based on Ct values. Primer pairs (Supplementary Table 2) were designed using the Primer 5.0 software. The *rpl17* gene was selected as the internal reference gene (Jin et al., 2021).

The expression of exosomal proteins SsMIF and SsPEBP1 in the ovary of black rockfish was obtained from proteome data previously determined by our laboratory (MOE Key Laboratory of Marine Genetics and Breeding) (Li et al., 2024b). A heat map depicting the expression of SsMIF and SsPEBP1 in the ovarian proteome was generated using TBtools software.

2.5. Immunohistochemistry

The ovarian tissue fixed in PFA was cut into 1 cm^3 pieces, dehydrated with graded ethanol, and embedded in paraffin wax. After slicing, dewaxing and hydration were performed in xylene and gradient alcohol. Sodium citrate antigen retrieval was used, followed by three PBST washes, 5 min each. The tissue was then blocked with 5 % goat serum (HG-FBS-500, HAKATA) for 2 h. After blocking, an anti-MIF rabbit antibody (1:200) was added overnight. The next day, PBST was used again five times, and goat anti-rabbit antibody (1250) was added. Finally, DAB was used for color development in the dark, and samples were subsequently observed and photographed using a light microscope to visualize the immunohistochemical staining.

2.6. Characterization of exosomes

The extraction of exosomes followed a previously published method (Chen et al., 2024). We selected the ovaries of artificially inseminated female fish as the source for exosome extraction. After collecting the ovarian fluid, it was first centrifuged at 3000*g* for 10 min, and the supernatant was then centrifuged at 12000*g* for another 10 min. After passing through a 0.22 μm filter (SAINING Biotechnology), exosomes were isolated using an exosome extraction kit (Umibio (Shanghai) Co., Ltd.). The isolated exosome solution was assessed for vesicle size distribution and concentration using nanoparticle tracking analysis. Exosomes were diluted 10,000-fold in particle-free PBS and measured using a ZetaView S/N 21–734 (Particle Metrix, Meerbusch, Germany), with data analyzed using ZetaView software (version 8.05.14 SP7). To observe the morphological characteristics of the exosome vesicles, the exosome suspension was fixed in 4 % PFA for 1 h, followed by negative staining with 2 % uranyl acetate for 1 min at room temperature. Observations were made using a JEOL JSM-840 electron microscope (Japan).

2.7. Expression and purification of recombinant SsMIF and SsPEBP1

Using the forward primer (underlined EcoR I site) and the reverse primer (underlined *Xho* I site), the cDNA encoding the full-length SsMIF or SsPEBP1 was amplified via PCR (Supplementary Table 2). The amplified DNA fragment was subcloned into the pET-28a expression vector (Novagen), which had been previously digested with the same restriction enzymes. The constructed plasmid pET-28a/SsMIF or SsPEBP1 was then transformed into *Escherichia coli* BL21 (DE3; TransGen Biotech). The rSsMIF or rSsPEBP1 was induced and purified as described previously (Chen et al., 2021). The isopropyl β-D-1-thiogalactopyranoside (IPTG), to a final concentration of 0.1 mM, was added to induce recombinant protein expression. After further incubation at 19 °C for 12 h, the cells were harvested and sonicated on ice. The recombinant rSsMIF or rSsPEBP1 was purified according to the method described by the previous study (Li et al., 2022). The purity of rSsMIF or rSsPEBP1 was analyzed using SDS-PAGE on a 12 % gel, followed by Coomassie Brilliant Blue R-250 staining. A protein marker ranging from 13 to 180 kDa (EC1019, Shandong Sparkjade Biotechnology Co., Ltd.) was used to indicate the molecular weight of the purified protein. To express the recombinant thioredoxin His Tag (rTRX) as a control, *E. coli* BL21 (DE3) cells were also transformed with the pET-32a (+) plasmid (Novagen) and induced at 28 °C with 0.5 mM IPTG for 8 h. Protein concentration was determined using the BCA Assay Kit (Novoprotein, Shanghai, China).

2.8. Western blotting

Specifically, an equal volume of exosome-specific lysis buffer was mixed with the exosome solution and lysed on ice for 15 min. Equal amounts of protein were separated on a 12 % SDS-PAGE gel and then electrotransferred onto a polyvinylidene difluoride membrane (Amersham). After protein transfer, the membrane was incubated at room temperature for 2 h in a blocking solution. Subsequently, the membrane was incubated overnight at 4 °C with primary antibodies (anti-EEF2, 1:1000, Abcam; anti-CD81, 1:500, anti-HSP70, 1:2000, anti-MIF, 1:1000, anti-PEBP1, 1:1000, Bioss USA, China). The membrane was washed five times with PBS containing 0.1 % Tween-20 (PBST) for 5 min each to remove unbound antibodies. Afterward, the membrane was incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG antibody (Biodragon, China) (diluted 1:4000 in blocking buffer) for 1 h, followed by five more washes with PBST for 5 min each. The bands were visualized using Super ECL Plus (S6009L, US EVERBRIGHT, Suzhou, China) according to the manufacturer's instructions.

To verify that the MIF and PEBP1 proteins can be transferred to sperm, in vitro incubation of the purified proteins with sperm was performed, followed by Western blotting to detect protein accumulation in the sperm. Specifically, total proteins were extracted from sperm incubated with rSsMIF, rSsPEBP1, and rTRX proteins for 2 h. The procedures for gel electrophoresis, membrane transfer, and color development were carried out as described above. β-Tubulin (1:2000, Proteintech) was used as a reference protein for sperm in quantitative analysis.

2.9. Effect of MIF or PEBP1 on sperm morphology

To evaluate the impact of MIF and PEBP1 on sperm morphology, proteins were co-incubated 6 h with sperm (1×10^7 cells/mL), and sperm morphology were assessed using sperm morphology stain kit (Diff-Quik Method, Beijing Solarbio Science & Technology Co., Ltd.). Staining was performed according to the manufacturer's instructions. Briefly, sperm were washed twice with PBS and then fixed in fixative solution for 10 s. Subsequently, the samples were stained with Diff-Quik Solution I for 10 s, followed by removal of excess dye. Diff-Quik Solution II was then applied for 5 s, and excess dye was removed. Finally, the slides were rinsed 10 times with running water, and sperm morphology was examined under a microscope.

2.10. Effect of MIF or PEBP1 on sperm viability and ROS level

To evaluate the impact of MIF and PEBP1 on sperm viability, proteins were co-incubated with sperm at different time points (0, 6, 12, and 24 h), and sperm viability was assessed using Live and Dead Cell Double Staining Kit (KTA1001, Abbkine, Wuhan, China). Specifically, sperm were diluted in PBS buffer to a concentration of 1×10^7 cells/mL and incubated at 13 °C with rSsMIF, rSsPEBP1, and rTRX proteins. After incubation, the sperm were washed twice with PBS to remove excess protein, then resuspended in an equal mixture of LiveDye and NucleiDye, and incubated at 37 °C in the dark for 30 min. Samples were placed in confocal dishes (SAINING Biotechnology), and observed using a Nikon Eclipse Ti—U microscope (Nikon, Tokyo, Japan). For each group, and at least 300 sperm cells were counted, and the percentage of live cells out of the total cell count was defined as sperm viability.

Intracellular ROS levels are important indicators of oxidative stress and cellular health. In this study, ROS levels in sperm were assessed before and after treatment with recombinant proteins using a ROS detection kit (Beijing Solarbio Science & Technology Co., Ltd.). Sperm treated with PBS, rTRX, rMIF, or rPEBP1 were washed twice with PBS, followed by the addition of the fluorescent probe DCFH-DA. The samples were incubated at 37 °C for 30 min. After removing excess probe with PBS, fluorescence intensity was measured before and after protein stimulation at an excitation wavelength of 488 nm and an emission wavelength of 525 nm.

2.11. Effect of MIF or PEBP1 on sperm motility and intracellular calcium ion content

To assess the effect of proteins on sperm motility, we used computer-assisted sperm analysis (CASA) as referenced in previous studies (Niu

et al., 2023). Specifically, after incubating with proteins for 12 h, 10 μL of sperm from each group were mixed with 30 μL of fetal bovine serum (FBS) to activate them. FBS has been confirmed as an in vitro activator for black rockfish sperm. The total motility and sperm trajectory parameters, including curvilinear velocity (VCL), average path velocity (VAP), straight-line velocity (VSL), linearity (LIN), and wobble (WOB), were measured using the SCA 6.4.0.82 software. The image sampling frequency was set to 200 Hz, the analysis timeout to 15 s post-activation, and the preview time to 1 s. A 3 μL sample of the activated sperm suspension was placed in a sperm counting chamber (SAS Medical, China) and observed under a 40× objective lens (Nikon ECLIPSE E200, Japan). A Nikon DS-Fi camera (Japan) was used to record 25 fields of view for each sample, and the total motility of at least 400 sperm was analyzed.

Sperm cell lysates and calcium ion standard solutions were prepared according to the instructions of the calcium ion assay kit (Beyotime Biotechnology). Equal volumes of the standard solution and samples to be tested were added to a 96-well plate, with each sample measured in triplicate. After adding the chromogenic working solution to all standards and samples, the plate was incubated at room temperature for 10 min, protected from light. The absorbance at 575 nm was measured using a microplate reader, and a standard curve was generated to calculate the calcium content in the test wells.

2.12. Statistical analysis

Statistical evaluation of qRT-PCR data was performed using the two-tailed Student's *t*-test in GraphPad Prism software version 6.0. For multiple comparisons of sperm viability, motility, calcium ion content and ROS level across different time points and treatment groups, one-way analysis of variance (ANOVA) was applied, followed by Tukey's post-hoc test in SPSS 20.0, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$. All data were expressed as mean ± SEM. Measurements were carried out in triplicates.

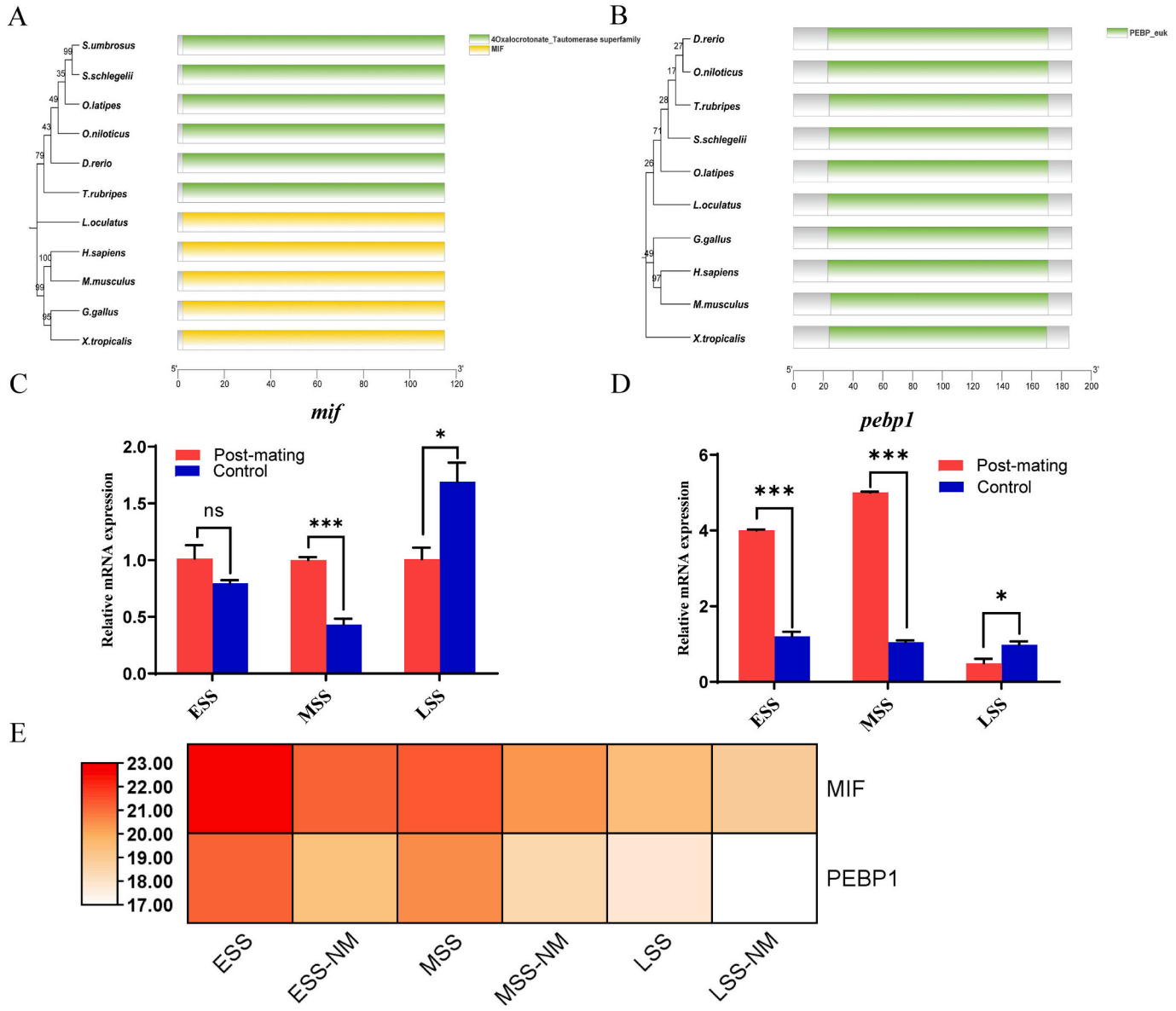

Fig. 1. Evolutionary analysis of *mif* and *pebp1* and its expression in the ovary of black rockfish. (A) The phylogenetic tree and a comparison of the protein domains of the *mif* gene across various species. (B) The phylogenetic tree and a comparison of the protein domains of the *pebp1* gene across various species. The numbers on the branches (e.g., 99, 35) represented bootstrap values, and the bars represented the presence and length of specific protein domains. Relative mRNA expression levels of *mif* (C) and *pebp1* (D) in ovarian tissues at different developmental stages (ESS, MSS, LSS) between post-mating and control groups. (E) Protein levels of MIF and PEBP1 in ovarian tissues at ESS, MSS, LSS stages between post-mating and control groups.

3. Results

3.1. Sequence identification and analysis of mif and pebp1

A phylogenetic tree and a comparison of protein domains in the *mif* or *pebp1* gene across various species were performed to investigate its evolutionary relationships. The phylogenetic tree showed distinct clusters corresponding to the evolutionary history of different species. For the *mif* gene, fish species (*S. umbrosus*, *S. schlegelii*, etc.) formed one cluster, while mammals (*H. sapiens*, *M. musculus*) formed another. Notably, all fish species, except for *L. oculatus*, possessed the 4Oxalocrotonate_Tautomerase superfamily domain, while mammals and amphibians exhibited the MIF domain (Fig. 1A). The consistent presence of both the MIF and 4-Oxalocrotonate Tautomerase domains across species underscores the evolutionary conservation of these domains, suggesting their functional importance in MIF biology. For the *pebp1* gene, fish species (*D. rerio*, *O. niloticus*, *T. rubripes*) clustered together, while mammals (*H. sapiens*, *M. musculus*) formed a separate cluster. The amphibian *X. tropicalis* formed a distinct branch, reflecting its unique evolutionary trajectory. All species analyzed contained the PEBP_euk domain, and its length was relatively consistent across different species, indicating a conserved function (Fig. 1B).

3.2. Upregulation of MIF and PEBP1 at mRNA and protein levels in post-mating

qRT-PCR was used to validate the expression levels of *mif* and *pebp1* genes in ovarian tissues. Compared to the non-mating group, the expression level of *mif* was significantly upregulated during the MSS period but showed a significant downregulation trend during the LSS period (Fig. 1C). Unlike *mif*, *pebp1* expression was significantly upregulated during the ESS period and continued into the MSS period. Similarly, *pebp1* expression was significantly downregulated during the LSS period (Fig. 1D). Additionally, using ovarian proteome data from the ESS, MSS, and LSS periods, it was found that the expression levels of MIF and PEBP1 proteins were higher in all three periods compared to the non-mating control group (Fig. 1E).

3.3. MIF and PEBP1 in exosomes from post-mating ovarian fluid

For the characterization of exosomes, on one hand, the study confirmed that exosomes from ovarian fluid were positive for CD81, HSP70, and EEF2 (Fig. 2A). On the other hand, transmission electron microscopy observed that the exosome particles were in the range of 80–160 nm and appeared as round or oval membrane-bound extracellular vesicles (Fig. 2B). Additionally, NTA analysis indicated that the concentration of the exosome solution extracted was 4.2 × 10^10 particles/mL, with an average particle size of 135.4 nm, and vesicles in the range of 80–160 nm accounted for 64 % of the total (Fig. 2C). Importantly, the study found that MIF and PEBP1 can serve as exosomal proteins in ovarian fluid (Fig. 2A), potentially playing a regulatory role in the ovarian microenvironment or on sperm.

3.4. MIF and PEBP1 in ovarian follicular and epithelial cells

MIF protein was primarily expressed in follicular cells and epithelial cells, with no significant differences in expression across the three stages. The expression location of the PEBP1 protein was consistent with that of MIF (Fig. 3).

3.5. Binding interaction of rMIF and rPEBP1 with sperm

The purified rMIF was successfully eluted at higher imidazole gradients (150, 200, and 250 mM), showing a single band with correct molecular weight (Fig. 4A). Similarly, the purified rPEBP1 and rTRX were successfully eluted at lower imidazole gradients (50, 70, and 90 mM), also displaying single bands with the correct molecular weights (Fig. 4B, C). Additionally, the reliability of the purified proteins was confirmed using His-tag antibodies (Fig. 4D). Following a 1-h incubation of rMIF and rPEBP1 with sperm, the presence of MIF and PEBP1 in the sperm was detected. After 3 h, their expression levels further increased. The differences in expression levels of both proteins before and after incubation demonstrated that both proteins could bind to sperm. As a control, the rTRX protein showed no expression in sperm before and after incubation (Fig. 4D).

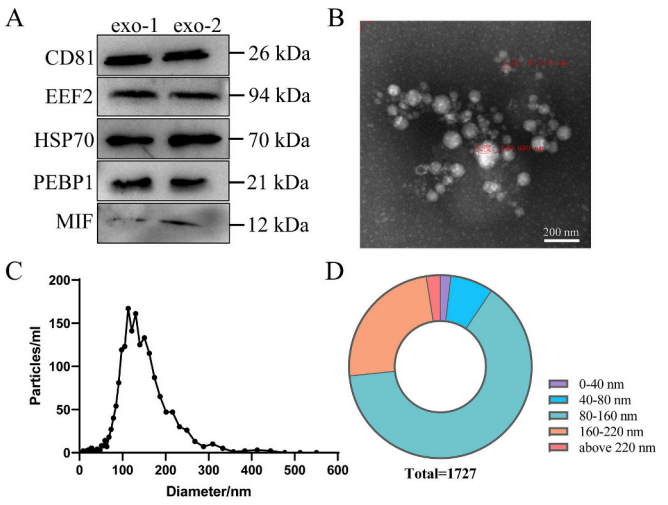

Fig. 2. Characterization of exosomes isolated from ovarian follicular fluid. (A) Western blot analysis of exosomal markers and proteins of interest in two exosome preparations (exo-1, exo-2). (B) Transmission electron microscopy (TEM) image of exosomes, showing their characteristic size and morphology. Scale bar: 200 nm. (C) Nanoparticle tracking analysis (NTA) showing the size distribution and concentration of exosomes. (D) Pie chart depicting the size distribution of exosomes.

3.6. The effects of recombinant proteins on sperm morphology

The effects of recombinant proteins on sperm morphology were analyzed, and the results are presented in Fig. 5. Both the rMIF and rPEBP1 treatment groups showed a significant increase in the percentage of normal sperm morphology compared to the PBS or rTRX group. Specifically, the sperm heads exhibited significant wrinkling or rupture, and the flagella were lost in the PBS or rTRX treatment groups. In contrast, sperm heads and flagella in the rMIF or rPEBP1-treated groups remained more intact. These results suggest that rMIF and rPEBP1 significantly improve sperm morphology, while rTRX has no significant effect.

3.7. Regulatory effects of rMIF and rPEBP1 on sperm viability and ROS levels

This study further investigated the regulatory effects of the two proteins on sperm. The results showed that sperm viability significantly increased after incubation with rMIF and rPEBP1 proteins, whereas the rTRX protein had no effect. Specifically, after 24 h of incubation with rMIF and rPEBP1, the sperm viability remained at 88 % and 85 %, respectively, while the viability in the control group dropped to 58 % (Fig. 6A, B). Furthermore, the intracellular ROS levels in sperm treated with these proteins were significantly lower than those in the tag protein and PBS control groups (Fig. 6C). These results indicate that MIF and PEBP1 can improve sperm viability in vitro, with MIF having a slightly stronger effect than PEBP1.

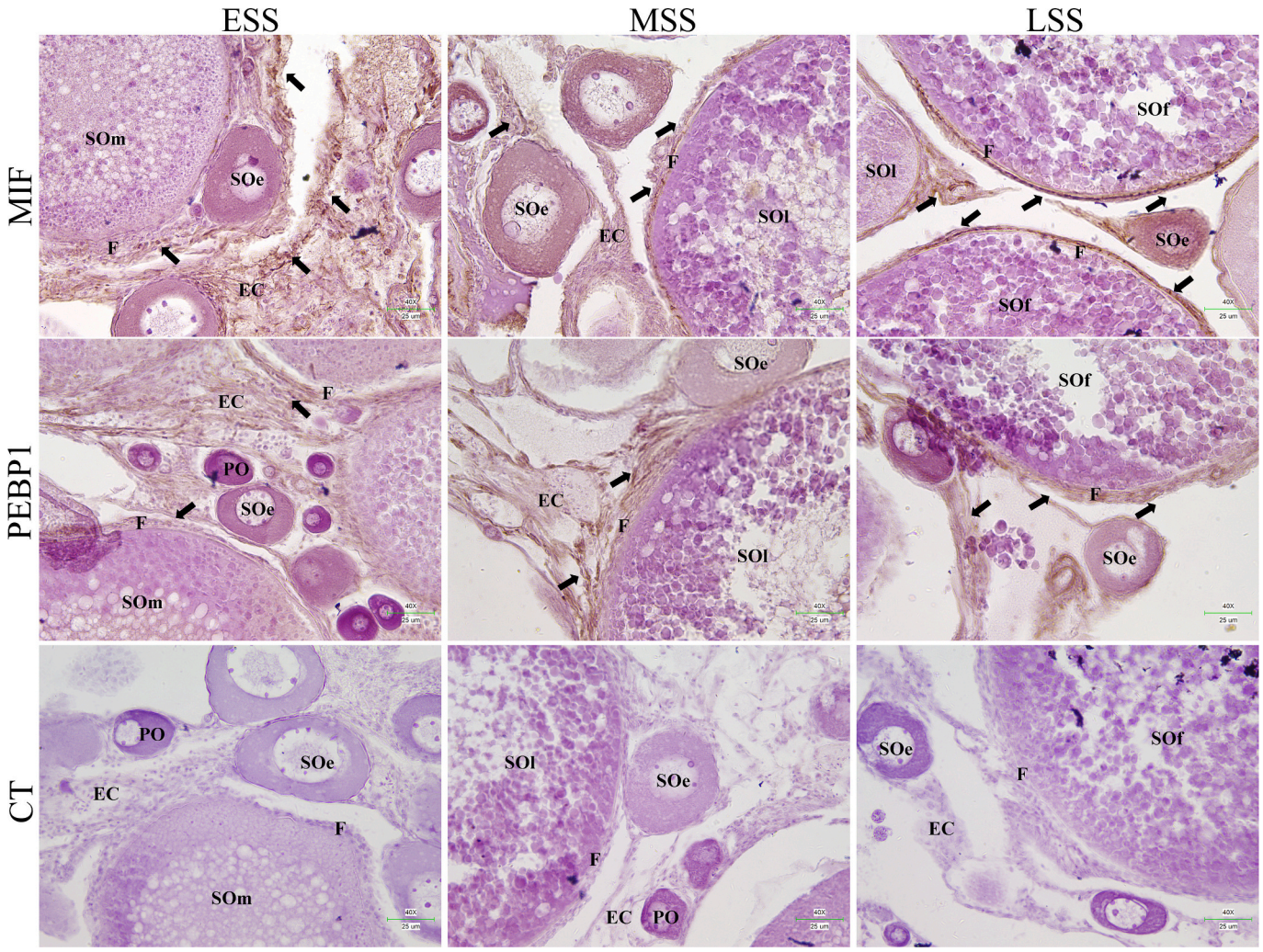

Fig. 3. Immunohistochemical staining of ovaries during ESS, MSS, and LSS stages (sperm-storage), which shows a positive expression of MIF and PEBP1. Arrows indicated positive signals. F, follicular layer; EC, epithelial cell; PO, primary oocyte; SOe, secondary oocyte early; SOm, secondary oocyte metaphase; SOl, secondary oocyte late; SOf, secondary oocyte final stage. Scale bars: 25 μm.

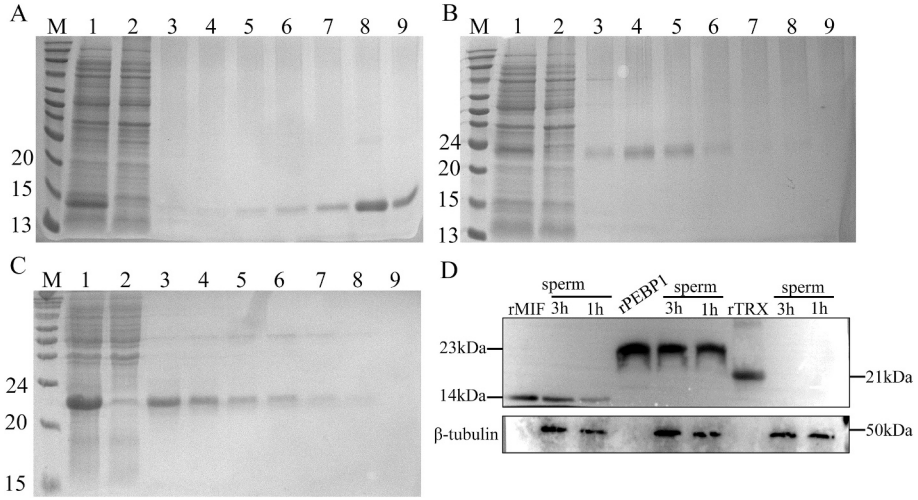

Fig. 4. The purification of recombinant MIF and PEBP1 proteins and transfer to sperm detection. (A) Elution profiles of purified recombinant MIF (A), PEBP1 (B), and TRX (C) proteins using varying concentrations of imidazole. Lane M: Protein marker (13–180 kDa); Lanes 1–9: IPTG induction, post-nickel column, 50 mM, 70 mM, 90 mM, 110 mM, 150 mM, 200 mM, and 250 mM imidazole elution. (D) Detection of His-tag in sperm after incubation with recombinant proteins for 1 h and 3 h. Purified recombinant proteins alone served as positive controls.

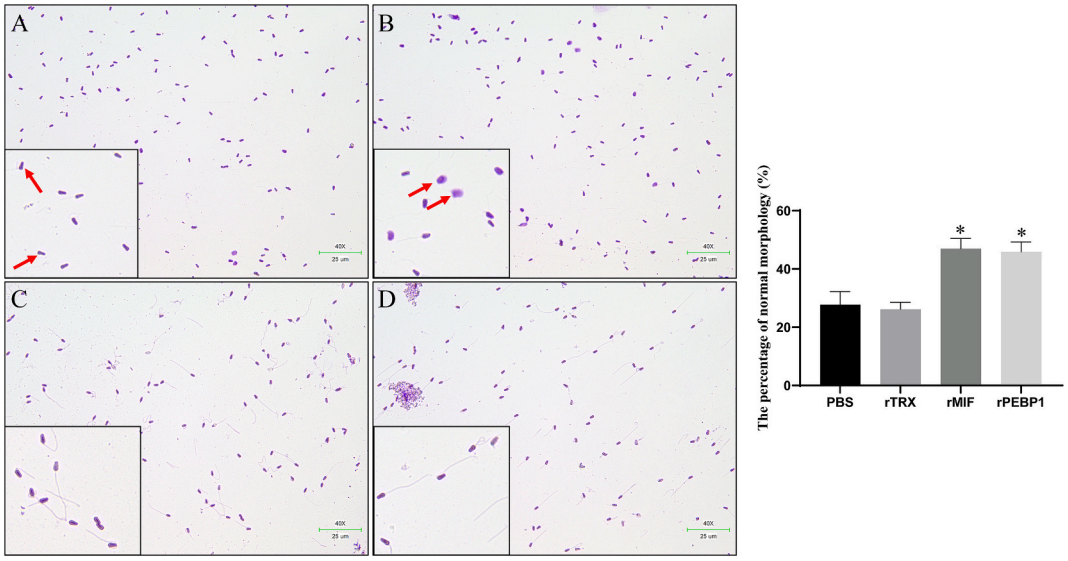

Fig. 5. Effects of recombinant proteins on sperm morphology. Representative images (A-D) show sperm morphology under different treatment conditions (PBS, rTRX, rMIF, and rPEBP1, respectively), with images captured at 40× magnification (scale bar = 25 μm). Arrows indicate damaged sperm. The bar graph on the right quantifies the percentage of sperm with normal morphology across the four groups.

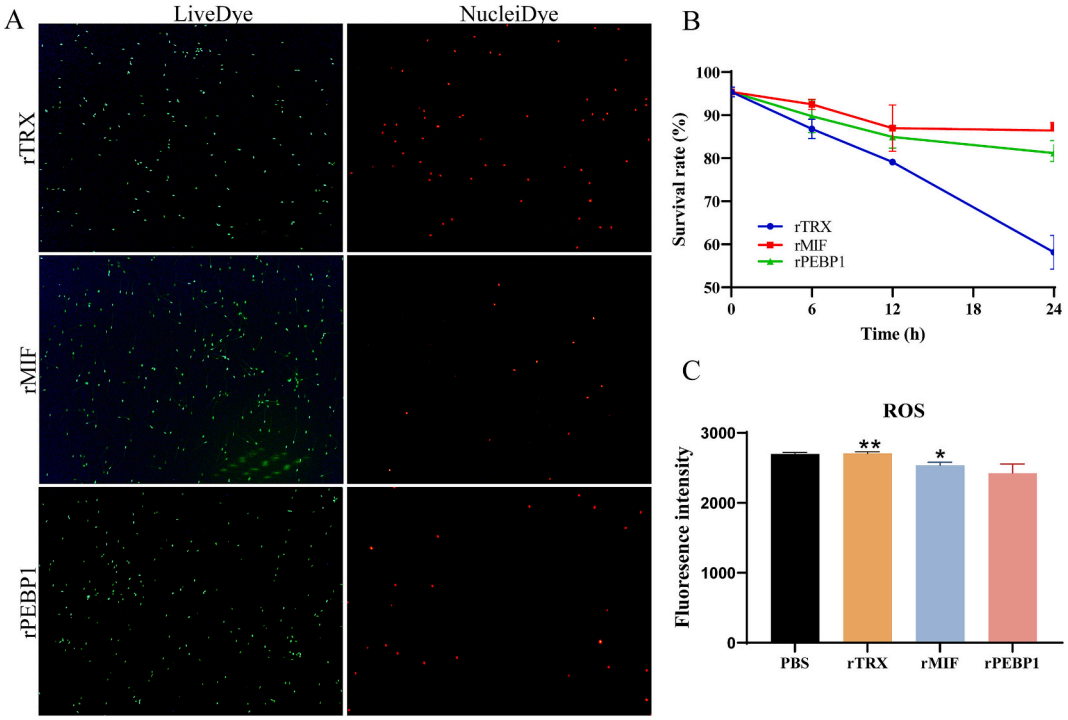

Fig. 6. Effects of recombinant proteins on sperm viability and ROS level. (A) Representative images of sperm viability assessed by Live/Dead staining after treatment with rTRX, rMIF, and rPEBP1 proteins. (B) Survival rate of sperm over time (0, 6 h, 12 h, 18 h, and 24 h) after incubation with rTRX, rMIF, and rPEBP1. (C) Sperm ROS levels after treatment with PBS (control), rTRX, rMIF, and rPEBP1.

3.8. rMIF and rPEBP1 improve sperm motility and increase intracellular calcium levels

Maintaining sperm motility is essential for their long-term storage. The results showed that percentage of motile cells in the rMIF and rPEBP1 incubation groups remained at 54 % and 60 %, respectively, compared to 40 % in the control group. Additionally, the motility parameters VCL, VAP, VSL, LIN, and WOB for sperm incubated with rMIF were 62.3 μm/s, 20.1 μm/s, 32.5 μm/s, 56.4 %, and 56.9 %, respectively, while those for sperm incubated with rPEBP1 were 57.4 μm/s, 25.4 μm/s, 35.2 μm/s, 62.8 %, and 62.9 %, respectively (Fig. 7A). Furthermore, the calcium ion content in sperm treated with rMIF and rPEBP1 was 43 μM and 42 μM, respectively, while the content in the rTRX and PBS groups was only 30 μM (Fig. 7B). These results indicate that both proteins effectively maintain sperm motility, adequately preparing them for subsequent fertilization.

4. Discussion

The female reproductive tract may mount an immune response to

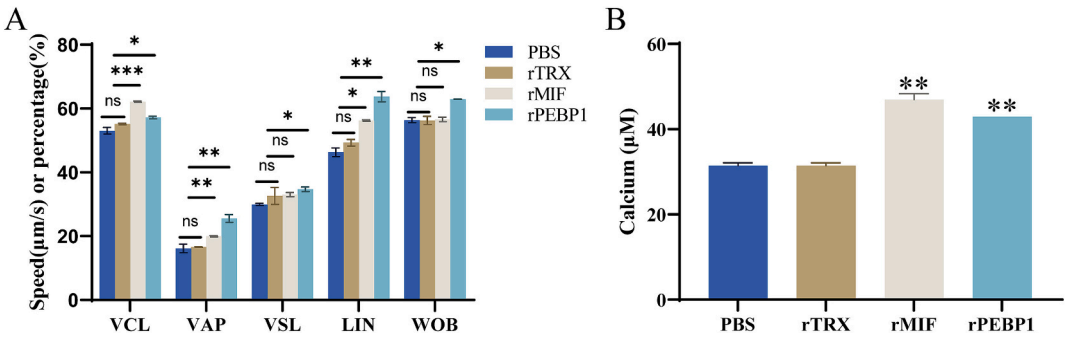

Fig. 7. Effects of recombinant proteins on sperm motility and intracellular calcium ion content. Sperm motility parameters (A) or intracellular calcium ion content (B) after treatment with PBS (control), rTRX, rMIF, and rPEBP1.

foreign cells, i.e., spermatozoa, and certain molecular and cellular mechanisms can protect sperm from being recognized and attacked by the female's immune system (Monin et al., 2020; Schjenken and Robertson, 2020). Additionally, the entry of sperm may induce changes in the microenvironment of the female reproductive tract, such as alterations in pH, ion concentration, and hormone levels, to optimize conditions for fertilization (Brotman et al., 2014). Due to the unique physiological structure of sperm, with minimal cytoplasmic content and organelles present in somatic cells, and with the sperm genome transcriptionally silent prior to fertilization, sperm development, and maturation are highly dependent on their microenvironment (Miyaso et al., 2022; Zhou et al., 2018). Our study found that after mating, the levels of MIF and PEBP1 were significantly upregulated following the entry of sperm. This may be evidence of the potential regulatory roles of MIF and PEBP1 on sperm presence, but it could also result from an immune inflammatory response (Buschow et al., 2017; Kang and Bucala, 2019; Roger et al., 2001; Yang et al., 2020). However, MIF and PEBP1 were expressed in the ovarian follicular and epithelial cells of black rockfish in ESS, MSS, and LSS three stages, which is consistent with the location of sperm storage. Furthermore, the secretion of exosomes at the sperm storage site has been observed in black rockfish (Chen et al., 2024) and other species (Chen et al., 2016; Riou et al., 2020; Waqas et al., 2017), which can facilitate prolonged sperm storage.

Notably, research indicates that after sperm enters the female reproductive tract, the ovaries may increase the release of exosomes (Al-Dossary et al., 2013; Chen et al., 2023). These exosomes can carry various proteins, RNA, and other biomolecules to support sperm survival (Riou et al., 2020), activation, and the fertilization process (Merc et al., 2021). This study confirmed that exosomes derived from the ovarian fluid of black rockfish are rich in MIF and PEBP1, and can be transferred to sperm. These results are consistent with findings from studies on mammals (Sullivan et al., 2005). Previous studies have also shown that some of these proteins, such as P25b, HE5 (CD52), SPAM1, and MIF, are associated with small membrane vesicles secreted by epididymal epithelial cells into the epididymal lumen, known as epididymosomes (Eickhoff et al., 2001; Frenette and Sullivan, 2001; Rejraji et al., 2006; Wanjari and Gopalakrishnan, 2023; Zhang and Martin-Deleon, 2003). In human seminal exosomes, AKR1B1 and PEBP1 can jointly regulate sperm status, maintaining them in a quiescent state (Zhou et al., 2021). In the whole proteome of buffalo sperm, the most enriched biological process was sexual reproduction, involving PEBP1, fetuin-B (FETUB), and acrosin (ACR) (Binsila et al., 2021).

Exosomes derived from the reproductive tract, can carry essential proteins and enzymes required for sperm maturation, such as sperm membrane-binding proteins, receptors, and ion channels, facilitating sperm functionality (Li et al., 2024a; Nixon et al., 2019; Saadeldin et al., 2014; Zhang et al., 2020). As a species with the characteristic of long-term sperm storage in females, the black rockfish provides an excellent biological model for studying the regulatory roles of exosomal proteins in the female reproductive system. This study confirms that in vitro incubation with the exosomal proteins MIF and PEBP1 can improve the number of morphologically intact sperm cells and their viability, as well as increase their motility percentage and velocity. Exosomal proteins derived from the female reproductive system also have multiple regulatory effects on sperm. For instance, after entering the female reproductive tract, exosomes may carry immunoregulatory molecules that enable sperm to evade the immune system's recognition and attack, ensuring their survival within the female reproductive tract (Archana et al., 2019; Foster et al., 2016; Ibrahim et al., 2019). By regulating energy metabolism and ion flux, exosomes enhance sperm motility, capacitation, and the fertilization process (Sysoeva et al., 2021; Sysoeva et al., 2022). Exosomal proteins, such as CD9, can promote the binding of sperm to the egg surface, thereby increasing the success rate of fertilization (Harada et al., 2013).

In this study, we isolated and characterized exosomes from the ovarian fluid of black rockfish, and for the first time, confirmed the presence of MIF and PEBP1 within these exosomes. Additionally, localization studies revealed that MIF and PEBP1 are primarily expressed in follicular and epithelial cells in the ovaries, which correspond to sites of sperm storage and exosome secretion. Subsequently, recombinant proteins demonstrated the ability to bind to sperm and directly regulate sperm morphology, survival, reactive oxygen species levels, motility, and calcium ion content. However, the regulatory roles of MIF and PEBP1 in sperm appear to function in parallel, with each protein exerting independent regulatory effects on sperm physiology. Whether these two proteins modulate the same cellular signaling pathways or exert synergistic effects remains an open question that requires further investigation. These findings not only enhance our understanding of the role of exosomes derived from female tissue fluids in sperm regulation but also provide new insights into the mechanism of long-term sperm storage in the ovaries of black rockfish. Furthermore, this study expands the known biological functions of MIF and PEBP1 proteins.

CRediT authorship contribution statement

Ying Chen: Writing – review & editing, Writing – original draft, Investigation, Data curation. **Yu Men:** Investigation, Formal analysis. **Kai Yan:** Investigation, Formal analysis. **Wajid Ali:** Investigation. **Zibin Li:** Investigation. **Wenxiu Cai:** Investigation. **Yan He:** Resources. **Jie Qi:** Writing – review & editing, Methodology, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgment

The work was supported by the National Natural Science Foundation of China [No. 32273133 and No. 31970492] and High-performance Computing Platform of YZBSTCACC. In addition, thanks for the technical support provided by Shanghai Bioprofile Technology Company Ltd.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aquaculture.2024.741985.

Data availability

No data was used for the research described in the article.

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