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Progress in cryopreservation of marine molluscan spermatozoa: technological advances, challenges and aquacultural applications

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Abstract. Cryopreservation of spermatozoa is of strategic importance in aquaculture breeding, large-scale seed production, and the conservation of endangered species. While research on sperm cryopreservation in marine mollusks lags behind that of livestock and fish species, significant progress has been made in recent years, particularly for key economically valuable species such as oysters, mussels, scallops, pearl oysters, clams, and abalones. This review systematically addresses four key technical aspects of sperm cryopreservation in bivalves and gastropods: (1) optimization of extender formulations; (2) selection of cryoprotectants; (3) regulation of cooling rates during the freezing process; and (4) thawing temperature control. Existing research has demonstrated that a combination of physiological traits, environmental factors, and cryopreservation protocols influences sperm quality. However, critical knowledge gaps remain in understanding marine mollusk sperm cryopreservation. This article synthesizes recent advancements and offers theoretical and practical frameworks for standardizing cryopreservation protocols, aiming to enhance post-thaw sperm viability and promote sustainable management of molluscan genetic resources.

Keywords: aquaculture, marine mollusks, sperm, cryopreservation, genetic resource conservation

1. Introduction

According to the latest data released by the Food and Agriculture Organization of the United Nations (FAO), global fishery and aquaculture production reached a historic high of 223.2 million tons in 2022, marking a 4.4% increase from 2020. Significantly, aquaculture contributed 51.3% of the total seafood supply, surpassing capture fisheries for the first time and indicating a transformative shift in global seafood production dynamics [1]. As the leading producer, China accounts for 64% of the world's aquaculture output, with marine aquaculture covering approximately 20.74 million hectares, of which 61.24% is allocated to shellfish cultivation. The report indicate that China's total aquaculture output will increase to 78.74 million tons by 2034, representing a 10.7% rise compared to 2020, with an average annual growth rate of 1.0% [2]. In response to the increasing demand for sustainable aquaculture development, researchers have adopted genetic improvement strategies, including selective breeding and hybridization, to enhance economically desirable traits in cultured species. A plethora of empirical evidence has demonstrated the success in a wide range of aquatic organisms, including carp, catfish, salmon, squid, marine finfish, oysters, and shrimp. Consequently, these methodologies have the potential to enhance the productivity of aquaculture and reinforce the genetic robustness of cultivated species [3, 4].

Sperm cryopreservation has been recognized as an effective strategy for the long-term preservation of biological material, offering three principal advantages. Firstly, it overcomes reproductive seasonality, thereby ensuring a stable and continuous supply of viable sperm. Secondly, it facilitates the establishment of secure genetic repositories for valuable strains, thereby reducing the risk of genetic erosion caused by disease outbreaks or environmental disasters. Thirdly, it is conducive to the secure and regulated transregional dissemination of superior genetic material in accordance with rigorous biosecurity protocols [5-8]. This technology has been extensively implemented in livestock and poultry breeding programs and. Indeed, it is now widely considered to be indispensable for the genetic management of high value species such as cattle and horses [8].. In contrast, within aquaculture, sperm cryopreservation efforts have primarily concentrated on teleost fish, with established cryopreservation protocols currently available for approximately 200 species [9].

Over the past decade, research on sperm cryopreservation in marine mollusks, particularly in important economic animals such as oysters, scallops, and abalones, has grown substantially [4, 10-12]. The process of sperm cryopreservation typically involves several key steps: assessment of sperm quality, sperm collection, diluent preparation, cryoprotectant preparation, cooling procedures, long-term storage, thawing, and post-thaw evaluation. Current studies in molluscan cryobiology primarily focus on optimizing extender compositions, selecting effective cryoprotectants, establishing appropriate cooling rates, and regulating thawing conditions. Key parameters used to evaluate cryopreservation outcomes include sperm motility, morphological integrity, fertilization potential, ultrastructural preservation, and organelle function [13]. A comprehensive grasp of these parameters is critical to enhance cryopreservation protocol reliability and post-thaw viability in marine mollusks.

2. Extender solutions

The standardized collection of sperm in the context of marine mollusks is typically undertaken through two primary methodologies: natural spawning and artificial extrusion. Natural spawning is a prevalent reproductive strategy among species such as mussels (Mytilus spp.), abalones (Haliotis spp.), and scallops (Chlamys spp.). However, this method is susceptible to contamination by mucus, seawater, or fecal matter, which often results in lower sperm concentrations and variable quality. Conversely, artificial extrusion-a technique frequently employed in the cultivation of oysters and pearl oysters-has been shown to be a relatively efficient and controlled method that yields higher sperm concentrations with reduced contamination. However, it should be noted that this approach may result in a greater degree of variability in the maturity and quality of the samples obtained [13, 14].

In studies of sperm cryopreservation in marine mollusks, the selection of an extender solution is closely associated with the sperm collection method. For sperm obtained through natural spawning, filtered and sterilized natural seawater is typically employed as the extender, whereas artificial seawater is rarely used, except in certain investigations involving small abalones (Haliotis spp.) [15]. Importantly, sperm collected via natural spawning is already in an activated state, initiating immediate metabolic processes such as flagellar movement and mitochondrial respiration, which rapidly deplete cellular energy reserves. Consequently, prolonged suspension significantly diminishes fertilization potential [15, 16]. Although storage at low temperatures (e.g., 4 °C) can temporarily preserve sperm viability, a more comprehensive understanding of sperm activation mechanisms, alongside the development of extenders designed to inhibit premature motility post-spawning, may conserve metabolic reserves and prolong cellular viability, ultimately contributing to improved post-thaw performance.

Artificial extrusion commonly employs calcium-free Hanks' Balanced Salt Solution (Ca^{2+}-free HBSS) as the extender. This solution has been reported to enhance not only sperm motility and fertilization capacity but also significantly increase larval yield. Additionally, modified extenders—such as the D solution, which is a tenfold dilution of HBSS—have demonstrated improved effectiveness in the cryopreservation of sperm from the triangular scallop (Chlamys nobilis) [17].

3. Cryoprotectants

Cryoprotectants are typically classified into two main categories based on their ability to permeate cell membranes: permeating cryoprotectants (e.g., dimethyl sulfoxide [DMSO], glycerol, and propylene glycol) and non-permeating cryoprotectants (e.g., sucrose, polyethylene glycol, and trehalose) [18]. Permeating cryoprotectants are defined by their minute molecular size and elevated membrane permeability. These substances rapidly diffuse into cells, thereby increasing the extracellular osmotic pressure and, in turn, promoting cell dehydration. This dehydration has been shown to reduce the amount of free water within the cell, thereby minimising the risk of ice crystal formation during the freezing process and effectively mitigating the cryoinjury caused by intracellular ice [19]. Conversely, non-permeating cryoprotectants remain extracellular and function primarily by elevating the osmotic pressure of the surrounding medium. The process of creating a hyperosmotic environment is pivotal in the context of cryopreservation, as it gradually draws water out of the cell. This, in turn, has the effect of reducing the likelihood of intracellular ice formation [20].

In the course of research conducted on the cryopreservation of Crassostrea gigas (Pacific oyster) sperm at ultra-low temperatures, a total of six cryoprotectants were subjected to evaluation, with each cryoprotectant assessed at five distinct concentrations. The optimal preservation protocol comprised 10% dimethyl sulfoxide (DMSO) as the cryoprotectant, calcium-free Hank's Balanced Salt Solution (HBSS) as the extender, a dilution ratio of 1:4, and supplementation with 0.45 M trehalose [11]. For Crassostrea virginica (American oyster) sperm, 10% and 15% propylene glycol (PG) were identified as the most effective cryoprotectants. Sperm samples that had been thawed from these treatments exhibited significantly higher levels of plasma membrane integrity and mitochondrial membrane potential in comparison to those samples that had been preserved with other concentrations [21, 22]. In the context of Patinopecten yessoensis spermatozoa, the presence of DMSO resulted in a substantial augmentation in cryoprotective efficacy when compared with glycerol and methanol. Optimal post-thaw motility occurred at 16% (v/v) DMSO, with motility positively correlated to concentration below this threshold but declining beyond 16% [19].

Antifreeze additives serve as auxiliary components in cryopreservation media, complementing extenders and primary cryoprotectants. Commonly used additives include sugars (e.g., trehalose, sucrose), amino acids (e.g., glycine, proline), and vitamins (e.g., vitamins E and C). These compounds exert cytoprotective effects through three main mechanisms: (1) inhibition of lipid peroxidation, (2) scavenging of reactive oxygen species (ROS), and (3) modulation of osmotic balance [23, 24]. Supplementation of 6% (v/v) DMSO with either 1% glucose or 2% sucrose significantly enhanced post-thaw viability in Haliotis laevigata (greenlip abalone) spermatozoa, improving membrane integrity by 23.7% and mitochondrial function by 18.4% versus DMSO alone. Notably, the addition of glucose improved sperm membrane integrity and mitochondrial membrane potential [25]. Moreover, supplementation with 0.6% glycine significantly increased the fertilization rate of thawed sperm [26], indicating a synergistic protective role of amino acids and sugars during cryopreservation.

4. Cooling rate

Sperm cryopreservation techniques in marine mollusks are broadly divided into two categories: non-programmed freezing (typically utilizing liquid nitrogen vapor) and programmed freezing. Both cryopreservation protocols seek to balance solute concentration effects against intracellular ice nucleation—actors governing post-thaw viability during freeze-thaw cycling [27].

Non-programmed freezing involves adjusting the vertical distance and exposure duration of sperm samples relative to the surface of liquid nitrogen, thereby establishing a gradient-based cooling process. This technique is characterized by its simplicity, cost-effectiveness and suitability for high-throughput applications, thus rendering it a practical choice for large-scale genetic resource preservation in aquaculture. Nevertheless, the instrument's limited precision compromises its applicability in research settings necessitating controlled conditions. In contrast, programmed freezing employs specialized equipment to execute pre-defined cooling protocols, allowing for precise regulation of cooling rates and high reproducibility. This method is particularly advantageous for experimental studies of cryopreservation under standardized conditions. However, it is constrained by higher costs, reduced sample throughput, and diminished practicality for commercial-scale operations.

Programmed freezing techniques employ programmable freezers to achieve precise regulation of the cooling process. The standard procedure generally encompasses the following: The first stage of the process is to set the final target temperature. The second stage is to select an appropriate cooling rate. The third stage, if applicable, is to incorporate an equilibration (or holding) phase. The fourth stage is to transfer the samples into liquid nitrogen for long-term preservation. As has been documented in previous studies, there is considerable interspecies variation in the optimal cooling parameters among marine mollusks. For instance, cryopreservation of Eastern oysters (Crassostrea virginica) utilized a terminal temperature of −30°C with a cooling rate of −2.5°C/min [22]. In contrast, investigations involving Pacific oysters (Crassostrea gigas), purple scallops (Argopecten purpuratus), and European oysters (Ostrea edulis) adopted lower final temperatures (ranging from −70°C to −100°C) and slower cooling rates (between −3°C/min and −8°C/min) [28, 29].

For the Japanese abalone (Haliotis discus hannai), sperm samples were exposed to liquid nitrogen vapor at a distance of 5 cm for 10 minutes, followed by immersion in liquid nitrogen for a minimum of 2 hours [12]. In the case of the Yesso scallop (Patinopecten yessoensis), spermatozoa were initially maintained at a distance of 20 cm above the liquid nitrogen surface for 3 minutes, then repositioned to 3 cm for an additional 10 minutes, resulting in a post-thaw sperm motility rate of 45% [30]. Additional studies have reported successful outcomes using variable cooling strategies, wherein samples were gradually cooled to temperatures between −30°C and −120°C before adjusting the distance to the liquid nitrogen surface [31-33]. It is imperative to continuously monitor temperature fluctuations during vapor-phase exposure in order to elucidate the effects of cooling rates on outcomes of sperm cryopreservation.

In certain studies, dry ice–methanol baths have been utilized as alternative cooling methods. To illustrate this point, sperm from the Pacific oyster (Crassostrea gigas) was subjected to a methanol bath for a duration of 10 minutes prior to being immersed in liquid nitrogen, resulting in post-thaw fertilization rates ranging from 80 to 90 percent [34, 35]. This technique, known as vitrification, has been explored for application in marine mollusks. However, outcomes have been inconsistent and generally suboptimal, indicating limited suitability for widespread implementation in these species.

5. Thawing temperature

In marine mollusks, thawing temperatures are typically classified into three categories: low (≤29 °C), moderate (30–49 °C), and high (≥50 °C) temperature ranges [13]. The thawing temperature plays a crucial role in determining post-thaw sperm viability and fertilization capacity. Studies on Pacific oysters (Crassostrea gigas) have demonstrated that different thawing temperatures yield significantly variable outcomes. For instance, sperm thawed at 20 °C [35] and 40 °C [18, 36] resulted in fertilization rates over 80%, in contrast to a marked decline to below 50% when thawed at 70 °C [37]. Overall, bivalve species generally exhibit improved post-thaw performance when exposed to moderate or low thawing temperatures.

In pearl oysters (Pinctada margaritifera), low-temperature thawing is routinely used. Specifically, thawing at 25 °C [32] and 27 °C [38] resulted in sperm survival rates of 86% and fertilization rates of 80%, respectively. In green-lipped mussels (Perna canaliculus), thawing at 18 °C yielded a fertilization rate of 80% [39]. In green-lipped abalones (Haliotis viridis), thawing

temperatures ranging from 40 °C to 80 °C produced varied fertilization outcomes. The highest fertilization rate (94.8%) was observed at 50 °C, followed by 40 °C. Thawing at 60 °C, 70 °C, and 80 °C also achieved satisfactory fertilization rates, all exceeding 80% [33].

6. Outlook

Sperm cryopreservation is a pivotal technology widely utilized in livestock breeding. Nonetheless, its application in marine mollusks remains in the early developmental phase. Although preliminary protocols have been established for certain economically important species—such as oysters and pearl oysters—these technologies have yet to gain widespread adoption in commercial aquaculture. Several barriers hinder their implementation, including the limited infrastructure in small-scale hatcheries (annual output <5 tons), and the early phase of selective breeding programs, which are still in the process of developing stable operational frameworks.

Most current investigations have centered on refining cryopreservation protocols, particularly in optimizing extenders, cryoprotectant formulations, and cooling dynamics. Yet, these protocols often exhibit limited reproducibility and suboptimal consistency, thereby hindering broader application. A key limitation is the insufficient attention paid to pre-freeze sperm quality, which plays a critical role in determining post-thaw outcomes. Furthermore, systematic studies on the structural and biochemical alterations occurring during the freezing and thawing processes are lacking, despite their central importance in assessing post-thaw viability and fertilization potential.

With ongoing advancements in cryobiology and a shift from empirical trial-and-error approaches to more mechanistic, molecularly guided strategies, sperm cryopreservation in marine mollusks is poised to play an increasingly central role in germplasm conservation, genetic improvement, and artificial reproduction. Integration of emerging technologies—including nanoformulated cryoprotectants, algorithm-driven freezing programs, and germline stem cell techniques—is projected to substantially enhance post-thaw sperm viability, from levels below 10% (prior to 2010) to over 60% by 2025.

Future developments must overcome interspecies physiological variability, scale-up bottlenecks, and emerging climate-induced stressors. Ultimately, the advancement of cryopreservation technologies will underpin the long-term sustainability of aquaculture, reinforce global food security strategies, and contribute to the conservation of marine biodiversity.

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