



Short communication

Improvement in non-programmable sperm cryopreservation technique in farmed greenlip abalone *Haliotis laevis*Yibing Liu^a, Xiaoxu Li^{b,c,*}, Tong Xu^d, Nicholas Robinson^{a,e}, Jianguang Qin^a^a School of Biological Sciences, The Flinders University of South Australia, Adelaide, South Australia 5042, Australia^b South Australian Research and Development Institute, West Beach, South Australia 5024, Australia^c Marine Innovation Southern Australia, West Beach, South Australia 5024, Australia^d Dalian Ocean University, Dalian 116023, China^e Nofima, PO Box 210, Ås, Norway

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ABSTRACT

This study assessed the effects of the addition of vitamins (L-ascorbic acid), amino acids (glycine and taurine), and monosaccharides (glucose, fructose and galactose) on sperm cryopreservation using a non-programmable freezing technique in farmed greenlip abalone. The results showed that the addition of taurine, glycine or L-ascorbic acid significantly improved the post-thaw sperm motility, whereas the post-thaw sperm fertilization rates were improved by the addition of glycine or L-ascorbic acid. Flow cytometry analysis demonstrated that the addition of glycine significantly enhanced the post-thaw sperm plasma membrane integrity and acrosome integrity. Results from the investigation on monosaccharides demonstrated that glucose, fructose and galactose had a similar cryoprotective effect, resulting in a similar level of the post-thaw sperm fertilization rate, plasma membrane integrity, mitochondrial membrane potential and acrosome integrity. In this study, the highest post-thaw fertilization rate of 96% was achieved by using the cryoprotective mediums containing 6% DMSO, 1% glucose and 0.6% glycine.

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1. Introduction

In Australia, genetic improvement programs, such as selective breeding and hybridization, have been established to improve the production and maintain the sustainable and competitive long-term development of the abalone aquaculture industry (Li, 2008). Recently, cryopreservation techniques have been developed and have potential for enhancing the efficiency of these programs by overcoming the asynchronous spawning between male and female in greenlip abalone *Haliotis laevis* on farm (Liu et al., 2014). In comparison with sperm collected from wild greenlip abalone (Zhu et al., 2014), those collected from farmed broodstock were more sensitive to cryopreservation. To achieve a post-thaw sperm fertilization rate of about 90% in farmed stocks, high sperm to egg ratios of 40,000:1 and 10,000:1 were required in programmable and non-programmable freezing techniques, respectively (unpublished data; Liu et al., 2014). For successful application of this technique in commercial hatchery production or genetic improvement programs, a higher fertilization rate is needed using a lower sperm to egg ratio. One of the aims of this study is to investigate whether higher fertilization rates using a lower sperm to egg ratio can be achieved with the addition of amino acids and vitamins when using the non-programmable freezing technique.

Sugars have been widely used as a part of sperm cryoprotective mediums in livestock and fish species. Sugars are thought to lead to more favourable osmotic pressures for the purpose of inducing sperm dehydration and reducing the incidence of intracellular ice formation, resulting in the maintenance of sperm quality during cryopreservation (Gómez-Fernández et al., 2012; Horváth et al., 2003). Glucose has been shown to play a positive role in sperm cryopreservation using the non-programmable freezing technique in farmed greenlip abalone (Liu et al., 2014). In other studies, fructose and galactose can provide better cryoprotection than glucose in canine (Ponglowhapan et al., 2004) dog (Yildiz et al., 2000) and red deer (Fernández-Santos et al., 2007). The effects of different types of monosaccharides on sperm cryopreservation have not been investigated in abalone and other marine mollusc species. Therefore, this study will investigate the effects of sugars, amino acids and vitamins on the success of cryopreservation for farmed greenlip abalone which may be applicable to other marine molluscs.

2. Materials and methods

2.1. Animals and gamete preparation

Three year old farmed animals were provided by the SAM Abalone, Port Lincoln, South Australia (SA) in early October and were transported by air to the Aquatic Sciences Centre, South Australian Research and

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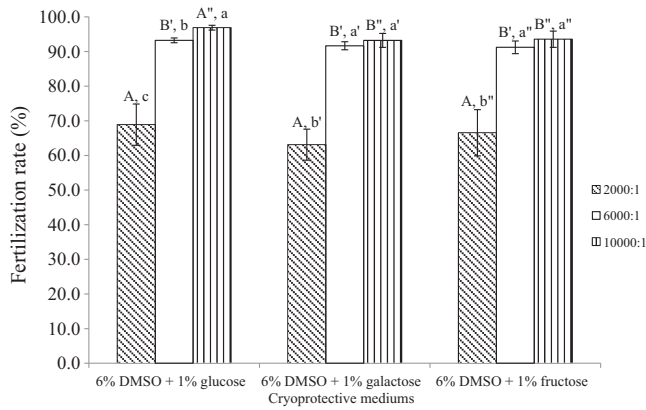


Fig. 1. Comparisons of post-thaw sperm fertilization rate (%) at different sperm to egg ratios in sperm cryopreserved in 6% DMSO plus different monosaccharides in farmed greenlip abalone ($n = 3$). Bars with different capital letters in each sperm to egg ratio indicate significant difference ($P < 0.05$) between cryoprotective mediums. Bars with different lowercase letters in each cryoprotective medium indicate significant difference ($P < 0.05$) between different sperm to egg ratios.

Development Institute (SARDI), Adelaide, SA. Methods for animal acclimation, spawning induction, and collection of concentrated sperm were described by Liu et al. (2014). Eggs were gently poured through a 300 μ m sieve and retained on a 90 μ m sieve at the bottom. The collected eggs were gently rinsed with 5 μ m filtered seawater and then washed into a settlement beaker. After 15 min the eggs on the bottom were transferred into another container and adjusted to a density of 1×10^4 mL⁻¹. The eggs were used in the subsequent experiments in 2 h post spawning.

2.2. Chemicals

Dimethyl sulfoxide (DMSO), glucose, fructose, galactose, glycine, taurine and L-ascorbic acid were in AR grade and purchased from Sigma-Aldrich Pty Ltd. Their stock solutions were prepared using 5 μ m filtered seawater at a double concentration as required in the experiments. When the stock solution was mixed with sperm at a 1:1 ratio (v/v), the final concentration was reached.

The LIVE/DEAD sperm viability kit (L-7011) for plasma membrane integrity (PMI) evaluation and LysoTracker green DND-26 (LYSO-G) kit (L-7526) for acrosome integrity (AI) evaluation were purchased from Invitrogen Australia. Rhodamine 123 (Rh 123) for mitochondrial membrane potential (MMP) evaluation and propidium iodide (PI) used for AI and MMP evaluation were purchased from Sigma-Aldrich Pty Ltd. The working solutions of these fluorescent agents were prepared according to Liu et al. (2014).

Table 1

Comparison of plasma membrane integrity, mitochondrial membrane potential and acrosome integrity between sperm cryopreserved in 6% DMSO plus different monosaccharides in farmed greenlip abalone ($n = 3$).

Cryoprotective mediums	Sperm component and organelle		
	PMI (%)	MMP (%)	AI (%)
6% DMSO + 1% glucose	39.3 ± 5.8% ^a	43.6 ± 7.4% ^a	47.7 ± 0.6% ^a
6% DMSO + 1% fructose	36.9 ± 5.5% ^a	44.7 ± 1.1% ^a	43.8 ± 5.8% ^a
6% DMSO + 1% galactose	37.1 ± 3.8% ^a	38.7 ± 4.4% ^a	46.4 ± 1.4% ^a

Same letter within each assessment parameter indicates that the difference is not significant ($P > 0.05$).

Table 2

Post-thaw sperm fertilization rates (%) between sperm cryopreserved in selected cryoprotective mediums in farmed greenlip abalone ($n = 3$).

Cryoprotective mediums	Sperm to egg ratios		
	500:1	1000:1	2000:1
6% DMSO + 0.6% glycine + 1% glucose	68.2 ± 7.0% ^{A₁c}	81.1 ± 6.2% ^{A'₁b}	93.6 ± 1.6% ^{A''₁a}
6% DMSO + 0.6% glycine + 1% galactose	61.2 ± 8.8% ^{AB₁b'}	63.3 ± 10.8% ^{B'₁b'}	91.7 ± 1.2% ^{A''₁a'}
6% DMSO + 0.6% glycine + 1% fructose	50.6 ± 7.5% ^{B₁b''}	51.7 ± 3.8% ^{C'₁b''}	86.8 ± 3.1% ^{A''₁a''}

Different capital letters in each sperm to egg ratio indicate significant difference ($P < 0.05$) between cryoprotective mediums. Different lowercase letters in each cryoprotective medium indicate significant difference ($P < 0.05$) between different sperm to egg ratios.

2.3. Sperm cryopreservation and quality evaluations

The sperm cryopreservation protocol optimized by Liu et al. (2014) was applied in this study. Briefly, the pre-cold concentrated sperm were mixed directly with the pre-cold cryoprotective medium at a 1:1 ratio and stored on ice for 10 min. The mixture was then transferred into 0.25 mL straws and placed on a rack 5.2 cm above the surface of liquid nitrogen (LN) in a styrofoam box. The straws were exposed to LN vapour for 10 min before being stored in LN for at least 24 h. The straws were thawed in 60 °C and recovered in 18 °C seawater baths.

The motility rate was determined by diluting the sperm suspension 5 times with 5 μ m filtered seawater and then counting the number of active sperm out of 100 by two independent observers. Sperm moving forward progressively were counted as active sperm while those vibrating or not moving at all were counted as dead sperm.

The fertilization assessment was conducted in 10 mL tubes. During fertilization, the required volume of concentrated eggs was taken using a pipette and mixed gently with post-thaw sperm to reach the specific sperm to egg ratio required in experiments. After contact for 10 min, the eggs were washed gently on a 90 μ m sieve by 5 μ m filtered seawater prior to being cultured in a 500 mL container. The fertilization rate was determined microscopically 4 h post-fertilization by counting the number of fertilized eggs out of a sample of 100. Eggs with two or multiple cells were counted as fertilized eggs and the rest was counted as unfertilized eggs.

For PMI, MMP and AI evaluations, thawed sperm were diluted to 2×10^6 sperm/mL and 1 mL of diluted sperm was then stained fluorescently prior to flow cytometric analysis. For PMI and MMP assessments, 100 μ L SYBR14 or Rh123 was added for 20 min, respectively, and then 100 μ L PI for a further 10 min. For AI analysis, 5 μ L LYSO-G was added for 30 min, and then 9 μ L PI for a further 10 min. All staining was carried out at the room temperature. The BD FACSVerse flow cytometer was used with 488 nm argon laser. The FACSuite software provided by the manufacturer was applied to calibrate the instrument settings. A total of 10,000 events were read in each sample. Density plots were generated

Table 3

Comparison of plasma membrane integrity, mitochondrial membrane potential and acrosome integrity between sperm cryopreserved in 6% DMSO + 0.6% glycine plus different monosaccharides in farmed greenlip abalone ($n = 3$).

Cryoprotective mediums	Sperm component and organelle		
	PMI	MMP	AI
6% DMSO + 0.6% glycine + 1% glucose	54.0 ± 5.1% ^a	50.8 ± 3.0% ^a	57.0 ± 1.5% ^a
6% DMSO + 0.6% glycine + 1% fructose	48.6 ± 2.2% ^a	48.5 ± 5.0% ^a	49.7 ± 6.1% ^a
6% DMSO + 0.6% glycine + 1% galactose	52.2 ± 4.7% ^a	46.8 ± 2.9% ^a	51.4 ± 6.8% ^a

Same letters within each assessment parameter indicate that difference is not significant ($P > 0.05$).

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