Preservation of Spermatogonia cell in Shrimp

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บทคัดย่อ

กุ้งเป็นกลุ่มทรัพยากรที่น่าสนใจเป็นอย่างมาก อีกทั้งยังเป็นหนึ่งในสินค้าทางการประมงที่มีการค้าขายกันอย่างแพร่หลายในตลาดต่างประเทศ และเป็นสินค้าหนึ่งในไม่กี่ชนิดที่ถือได้ว่าเป็น "สินค้าโภคภัณฑ์" ซึ่งก่อให้เกิดประโยชน์ทางเศรษฐกิจเป็นอย่างมากโดยเฉพาะอย่างยิ่งในประเทศที่กำลังพัฒนา ปัจจุบันการเลี้ยงกุ้งในประเทศไทยเช่นกุ้งก้ามกราม กุ้งแชบ๊วย และกุ้งกุลาดำมีความสำคัญทางเศรษฐกิจเป็นอย่างมาก ซึ่งในขณะนี้จำนวนกุ้งเหล่านี้ที่มีอยู่ในธรรมชาติมีจำนวนลดลง ดังนั้นการอนุรักษ์สายพันธุ์กุ้งจึงมีความสำคัญในการป้องกันการสูญพันธุ์ วิธีการแช่เยือกแข็งเป็นวิธีการที่ถูกใช้ในการเก็บรักษาเซลล์สืบพันธุ์ของสัตว์หลายชนิดที่ใกล้สูญพันธุ์ แต่อย่างไรก็ตามยังไม่มีรายงานในการเก็บรักษาเซลล์สืบพันธุ์เพศผู้ (spermatogonia cell) ของกุ้งโดยวิธีการแช่เยือกแข็ง ดังนั้นในการวิจัยครั้งนี้จึงมีวัตถุประสงค์เพื่อระบุระยะการพัฒนาเซลล์สืบพันธุ์เพศผู้สำหรับใช้ในกระบวนการเก็บรักษาด้วยการแช่เยือกแข็ง หาปัจจัยที่เหมาะสมสำหรับกระบวนการแช่เยือกแข็ง ได้แก่ เวลาที่ใช้บ่มในสารไครโอโพรเทกแทนต์สำหรับวิธีการแช่แข็งแบบช้าที่ 15, 30 และ 60 นาที ชนิดและความเข้มข้นของสารไครโอโพรเทกแทนต์ได้แก่ ไดเมททิล ซัลฟอกไซด์, กลีเซอรอล และแมกนีเซียมคลอไรด์ที่ความเข้มข้น 5, 10 และ 15 เปอร์เซ็นต์ และอุณหภูมิที่ใช้ในการละลายที่ 10 และ 27 องศาเซลเซียส

เพื่อรักษาเซลล์สืบพันธุ์ของกุ้งก้ามกราม กุ้งแชบ๊วย และกุ้งกุลาดำ โดยเปรียบเทียบระหว่างวิธีการแช่แข็งแบบช้าและแบบเร็วเพื่อการเก็บรักษาเซลล์สืบพันธุ์ให้คงอยู่ในระยะยาว ซึ่งเปอร์เซ็นต์การมีชีวิตรอดและการฟื้นตัวของเซลล์สืบพันธุ์เพศผู้ที่สดและผ่านการแช่เยือกแข็งของกุ้งจะได้รับการประเมินโดยการย้อมสีด้วยทริปแพนบลู

ผลการทดลองที่ได้จากการทดสอบเวลาที่เหมาะสมที่ใช้บ่มในสารไครโอโพรเทกแทนต์ (ไดเมททิล ซัลฟอกไซด์ หรือ กรีเซอรอล หรือ แมกนีเซียมคลอไรด์ที่ความเข้มข้น 10 เปอร์เซ็นต์) สำหรับวิธีการแช่แข็งแบบช้าของเซลล์สืบพันธุ์ของกุ้งก้ามกรามที่ถูกบ่มในอาหารสำหรับแช่เยือกแข็งและสารละลายน้ำเชื้อที่เวลา 15, 30 และ 60 นาที ไม่มีความแตกต่างกันอย่างมีนัยสำคัญทางสถิติ และผลการทดลองชนิดและความเข้มข้นสารไครโอโพรเทกแทนต์ทั้ง 3 ชนิด พบว่าสารที่มีเปอร์เซ็นต์การมีชีวิตรอดและการฟื้นตัวที่ดีที่สุดสำหรับเซลล์สืบพันธุ์เพศผู้ของกุ้งก้ามกรามและกุ้งแชบ๊วยคือ 10% DMSO และสำหรับเซลล์สืบพันธุ์เพศผู้ของกุ้งกุลาดำคือ 10% GLY อุณหภูมิที่ใช้ในการละลายที่ดีที่สุดคืออุณหภูมิที่ 10 องศาเซลเซียสสำหรับกุ้งก้ามกราม กุ้งแชบ๊วย และกุ้งกุลาดำ สำหรับการเก็บรักษาด้วยการแช่เยือกแข็งในระยะยาว การฟื้นตัวของเซลล์อสุจิที่เก็บรักษาไว้ในไนโตรเจนเหลวที่เวลา 6 เดือนด้วยวิธีการแช่เยือกแข็งแบบเร็วนั้นสูงกว่าการแช่เยือกแข็งแบบช้าในกุ้งทั้ง 3 ชนิดอย่างมีนัยสำคัญ และจำนวนเซลล์ทั้งหมดหลังจากที่เก็บรักษาโดยการแช่เยือกแข็งไม่เกิดการตายแบบอะพอพโทซิสระหว่างการเก็บรักษาด้วยความเย็นสำหรับทั้งสองวิธี การศึกษานี้เป็นหลักฐานว่าเซลล์สืบพันธุ์เพศผู้ในกุ้งก้ามกราม กุ้งแชบ๊วย และกุ้งกุลาดำ สามารถเก็บรักษาไว้ในไนโตรเจนเหลวได้ในระยะยาว ที่สำคัญการศึกษาของเราแสดงให้เห็นว่าการเก็บรักษาด้วยการแช่เยือกแข็งสามารถทำได้สำเร็จโดยไม่ต้องใช้อุปกรณ์พิเศษหรืออุปกรณ์ราคาแพง (ภาชนะแช่แข็งและช่องแช่แข็ง -80 องศาเซลเซียส) โดยใช้วิธีการแช่เยือกแข็งแบบเร็ว

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ABSTRACT

Prawns and shrimp are the most interesting group of resources, they are also one of the most widely traded fishery products on the international market and they are one of the few that can be considered a "commodity," generating significant economic benefits, especially for many developing countries. In Thailand, shrimp culture (such as giant freshwater prawn, banana shrimp and black tiger shrimp) is economically important of market. Nowadays, the number of shrimps in natural is decreased. Therefore, the conservation of shrimp species is important to prevention. Cryopreservation has been used to preserve germ cell of many endangered species. However, this method has not been established for cryopreservation of spermatogonia cell in shrimp. Therefore, in this study aimed to identify the spermatogonia cells developmental stage for cryopreservation and optimize the equilibration time (15, 30 and 60 min) for slow freezing method, type and concentration of cryoprotectants (dimethyl sulfoxide (DMSO), glycerol (GLY), and magnesium chloride ($MgCl_2$) at 5%, 10%, and 15%), and thawing temperature (10 and 27 °C) for preserve spermatogonia cell of giant freshwater prawn, banana shrimp and black tiger shrimp by compare between slow freezing and vitrification methods for a long-term preservation of spermatogonia cells. The viability and recovery percentage of fresh and frozen spermatogonia cell of shrimp were assessed by staining spermatogonia cell with trypan blue.

The result was obtained in equilibration time tested of giant freshwater prawn spermatogonia cells were incubated in cryomedium containing

cryoprotectants (10% DMSO, 10% GLY and 10% $MgCl_2$) and extender for 15, 30 and 60 min, there was not significantly difference. In next experiment, among three cryoprotectants tested, the best result of viability and recovery percentage was obtained with 10% DMSO for spermatogonia cell of giant freshwater prawn and banana shrimp and 10% GLY for spermatogonia cell of black tiger shrimp. The best thawing was found at 10 ºC for giant freshwater prawn, banana shrimp and black tiger shrimp. For long-term cryopreservation, the recovery of spermatogonia cells preserved in liquid nitrogen at 6 months with vitrification method were significantly higher than of preserved with slow freezing method in all shrimps and the total cells were observed after preserved not apoptosis during cryopreservation for both methods. This study provide evidence that spermatogonia cell in giant freshwater prawn, banana shrimp and black tiger shrimp can be preserved long-term in liquid nitrogen. Importantly, our study demonstrate that cryopreservation can be successfully performed without requiring no special or expensive equipment (freezing container and -80 ºC freezer) by using vitrification method.

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Natthida Rakbanjong

CONTENTS

LIST OF FIGURES

Figure **Page**

LIST OF ABBREVIATIONS AND SYMBOLS

BSA = Bovine serum albumin

cm = Centimeter

cDNA = complementary DNA

CPAs = Cryoprotectants

DEPC = Diethypyrocarbonate

DI = Deionized water

DMSO = Dimethyl sulfoxide

DW = Distilled water

EDTA = Ethylenediamine tetraacetic acid

FBS = Fetal bovine serum

g = Gram

GLY = Glycerol

h = Hour (s)

H_2O = Water

HCL = Hydrogen chloride

ISH = *In situ* hybridization

KCL = Potassium chloride

KH_2PO_4 = Potassium hydrogen phosphate

min = Minute (s)

M = Molar

$MgCl_2$ = Magnesium chloride

mM = Millimolar

ml = Milliliter

n = Number

ng = Nanogram

N = Number replicates

NaCl = Sodium chloride

NaOH = Sodium hydroxide

Na_2HPO_4 = Sodium hydrogen phosphate

PCR = Polymerase Chain Reaction

PGCs = Primodial germ cell

RNA = Ribonucleic acid

RT = Room temperature

RT-PCR = Reverse transcription polymerase chain reaction

s = Second (s)

S = Sperm

SC = Spermatocyte

SDS = Sodium dodecyl sulfate

SG = Spermatogonia cell

SSC = Spermatogonial stem cell

Tris-HCL = Tris (hydroxymethyl) aminoethane hydrochloric acid

°C = Degree Celsius

µg = Microgram

µl = Microliter

µm = Micrometer

µM = Micromolar

CHAPTER 1

INTRODUCTION

1.1 Background and Rationale

Prawns and shrimp are the most interesting group of resources, the life cycle is complicated, ranging from tropical estuarine habitats to shallow shelf environments, deep seas, and continental slopes. They are also one among the most popular widely fisheries products that have been traded on the international market, having a market capitalization of ten billion dollars (or 16 percent of global fishery exports), it is one of the few that can be considered a "commodity", producing substantial economic advantages, particularly for many developing markets. They make a significant contribution to the livelihoods of poor and vulnerable communities, particularly as a source of income (FAO. 2008). In Thailand, shrimp culture (such as Giant freshwater prawn, Banana shrimp and Black tiger shrimp) is important for economic with an output of 378,300 tons per year in 2016 reaching a market value of approximately 1875 million US$ (Fisheries Statistics of Thailand. 2018).

On the environmental field, climatic factors have a major impact on shrimp. However, coastal habitat loss is a common occurrence (FAO. 2008), such as aquaculture's destruction of mangroves or illegal trawling's destruction of seagrass beds in coastal areas, climate change, habitat destruction and life cycle of shrimp, etc. The number of shrimps in natural is decreased. Therefore, the conservation of shrimp species is important to prevention.

Cryopreservation is a well-known technique that uses liquid nitrogen (-196 °C) as a refrigerant to keep biomaterial samples working during long-term storage (Chao et al. 2009) and it can be used in biotechnology, animal husbandry, aquaculture, and endangered species conservation. (Golshahi et al. 2018). However, egg of aquatic

animals is the large size and high lipid and yolk content of eggs, it remains extremely difficult to cryopreservation (Yoshizaki and Lee 2018). Thus, in this research, we concentrated on spermatogonia cells which are sufficiently small (10-µm diameter) (Seki et al. 2017) and small enough to be cryopreserved. Also, spermatogonia cells which have the ability to develop into functional gametes (sperm and eggs) by transplanting them into a recipient of a species that is closely related to the donor. The recipient will be able to develop donor-derived gametes until they reached adulthood (Yoshizaki and Lee 2018) that has proven to be successful in a variety of fish species. Therefore, spermatogonia cells can be a viable alternative to gamete cryopreservation.

Although the cryopreservation method has already been used to successfully preserve fish spermatogonia cells, such as Nile tilapia (Higaki et al. 2018), rainbow trout (Lee et al. 2013), and common carp (Franěk et al. 2019), but not yet report mentioned successful cryopreservation of spermatogonia cell in shrimp. Therefore, this study aims to identify an optimal condition (equilibration time, cryoprotectant type and concentration and thawing temperature) and compare the cryopreservation efficiencies of slow freezing and vitrification methods for a long-term preservation of spermatogonia cells of shrimp.

1.2 Review of Literature

1.2.1 Giant freshwater prawn (*Macrobrachium Rosenbergii*)

Giant freshwater prawns are the common name of *Macrobrachium Rosenbergii* (De Man 1879), is the aquatic invertebrate from the palaemonidae, the crustacean class and the phylum is arthropoda of animal kingdom. Giant freshwater prawns are widespread in the tropical and subtropical seas of the Indo-Pacific area (Ling 1969), including Thailand, Malaysia, India, the Philippines, Myanmar, Indonesia, Sri Lanka, Bangladesh, and Vietnam. The majority of species that spend their early lives in saltwater are related to the sea in some way, giant fresh water Prawns are the most commonly cultivated of all the freshwater prawns (Chow et al. 1982) and widespread in regions of Southeast Asia. Thailand has been cultured continuously for a long time and can be cultured in all regions of the country because it is in demand of the market while in nature has decreased.

1.2.1.1 Biological and taxonomic characteristics

Giant freshwater prawns (*Macrobrachium Rosenbergii*) are the Macrobrachium species with the largest size. Males and females have maximum recorded sizes of 33 cm and 29 cm, respectively. The cephalothorax is divided into five indistinct segments in the head and eight in the thoracic region. The abdomen is divided into six movable terga segments. A pair of biramous pleopods are found in each abdominal segment (swimming legs). Two pairs of antennae, three pairs of jaws, three pairs of maxillipids, and five pairs of walking legs make up the cephalothorax. Laterally, the second and largest abdominal tergum overlaps the first and third abdominal tergum. Abdominal carapace has a smooth, rounded dorsal surface. The rostrum is long and slender, bent upwards. The rostrum has 8-10 ventral teeth and 11-14 dorsal teeth that reach beyond the antennal scale. Male prawns are much larger and thicker than females as they reach maturity, and the second pair of chelipeds is much larger

and thicker than in females. Males have a wider head and a smaller belly in comparison to females. Unlike some other *Macrobrachium* species, *M. rosenbergii* has equal-sized left and right chellipeds. They grow exceptionally long in adult males, reaching well beyond the rostrum's tip. The genital pores of males are found at the base of the fifth walking leg, whereas the genital pores of females are found at the base of the third walking leg. The telson's tip reaches beyond the spines of the posterior telson (Jayachandran 2001).

Figure 1: Giant freshwater prawns (*Macrobrachium Rosenbergii*)

Reference: (FAO. 2020).

1.2.1.2 Life cycle

Giant freshwater prawn (*Macrobrachium Rosenbergii*) populates in both brackish and fresh water, adult inhabit in fresh water and moved to brackish water in mating season because the larvae only develop and grown in brackish water. *M. rosenbergii* is a nocturnal, bottom-dwelling, and slow-moving species. They prefer shallow, detritus-rich, and vegetated areas during the day and remain half buried in sediments during the day (FAO. 2020). A freshwater prawn goes through four stages in its life: egg, larva, juvenile, and adult (Figure 2). Freshwater prawns, like other crustaceans, moult. The number of moults and intermoult durations are not defined and are affected by the climate, especially temperature and food availability.

The larvae of Giant freshwater prawn live on a non-permanent zooplankton when floating in the water, with the head below and the tail pointing upwards and move towards the light (Ling 1969). After hatching into larvae, the food of shrimp during this period is small organisms that float in the water such as small water rotifers, larvae of crabs and molluscs, etc. In this stage, young larvae float along the stream, so they often become food for the fish and there are few shrimp larvae that can survive and grow.

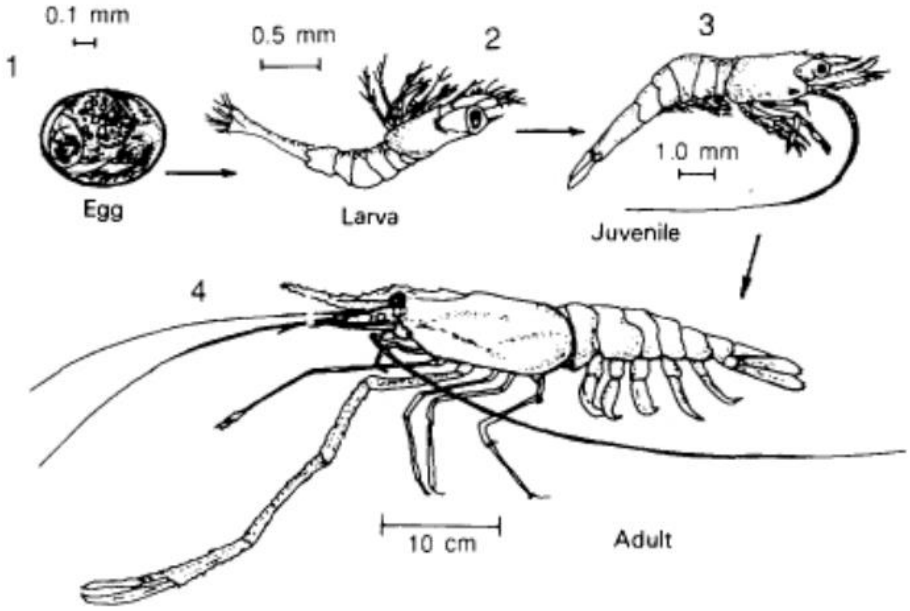


Figure 2: The life cycle of Giant freshwater prawns (*Macrobrachium Rosenbergii*)

Reference: https://www.slideshare.net/rnsImran/biology-of-prawn-*macrobrachium- rosenbergii*

1.2.2 Banana prawn (*Fenneropenaeus merguiensis*)

Banana prawn is the common name of *Fenneropenaeus merguiensis* (de Man, 1888), from the *merguiensis* species, *Penaeus* genus, the penaeoidea super family of the penaeidae family (penaeid prawns), the dendrobranchiata suborder of decapoda order, the malacostraca subclass of crustacean and the phylum is arthropoda of animalia kingdom. Banana shrimp (*Fenneropenaeus merguiensis*) is found in tropical and subtropical seas across the Indo-West Pacific area. In South-East Asia and Australia, *F. merguiensis* is a significant species for prawn fishing and substantial prawn farms. (Peterson and Daniels 1992).

1.2.2.1 General features and distribution

The *F. merguiensis*, white banana prawns have characteristics in common with other species of the genus *Penaeus* that has a high, toothed rostral crest and a poorly developed gastro-orbital ridge, shown in Figure 3. Their bodies are light yellow or transparent, with reddish brown spots strewn throughout them (Meager et al. 2003). The maximum recorded total length is 240 mm (approximately 60 mm carapace length) for female and 200 mm total length (approximately 50 mm carapace length) for males. Female banana prawns can weigh up to 69 g, males up to 59 g. Prawns have all the second abdominal side plates overlapping tile-like from the front which is different from the shrimp. Tough they are readily available in the wild, they can be produced in great densities since larval rearing is simple, and they thrive in both extensive and semi-intensive ponds, they tolerate a wide range of salinities and temperatures, they have poor protein requirements, and small size variations (Staples and Vance 1987).

Figure 3: Banana shrimp (*Fenneropenaeus merguiensis*)

Reference: Australian Institute of Marine Science.

Banana prawns inhabit tropical and subtropical waters. The preferred habitat of White banana prawns can be found in coastal waters ranging from shallow estuarine and intertidal waters to 45 meters deep. They spend the majority of their lives in turbid waters, swimming in muddy estuaries and on muddy beaches off the coast. In a sheltered mangrove area, juveniles live in small creeks and rivers with water ranging from almost fresh to highly saline (more than 70 parts per thousand). Adult white banana prawns prefer low-and medium-energy coasts, although they can survive high-energy cyclones. They favor normal (35 parts per thousand) or slightly less saline seawater than do juveniles. At high tide, *F. merguiensis* move into the mangrove forest, with more prawns found at the mangrove-creek edge (5-8 meters into the forest) than a further 25 meters into the mangrove forest. The reasons for this are uncertain, but food availability or tidal inundations are suggested (Meager et al. 2003).

1.2.2.2 Life cycle

Dall et al. (1990) and Yano (1993) revealed that the normal life cycle of a marine shrimp begins with adult organisms moving up to several kilometers offshore, developing, and spawning (Figure 4). The maximum life span for white banana prawns is 12- 18 months. Mating occurs in during molting. Prior to the next molt, the eggs are shed into the water and fertilized externally by sperm from the male's spermatheca. Individual prawns are partial spawners and each female can lay several batches of eggs each year. In captivity, banana shrimp generate 50,000-400,000 eggs every spawning, but in the wild, they produce 100,000-1,000,000 eggs per spawning. From egg to postlarvae, larval development is complicated and involves three stages: nauplius, protozoea, and mysis. Over the course of two to three weeks, larvae grow and molt from floating fertilized eggs. Juveniles like shallow salty water along marsh margins, where plants offer cover and debris, and microorganisms thrive in the softbottom (Rosenberry 2002). The juvenile growth rate is rapid in white banana prawns, with increases of approximately 1.2 mm carapace length per week (Stapels 1980). As an opportunistic omnivore on the sea floor, the adult shrimp continues to develop in size in the gulf, and its current niche is largely as a source of food for a large number of people. White banana prawns prefer coastal water, which varies in depth from shallow estuary and intertidal seas to 45 meters. Unfortunately, only a few adult shrimps can live long enough to reproduce when the water column temperature rises sharply, restarting the cycle by causing the females to spawn and produce thousands of eggs.

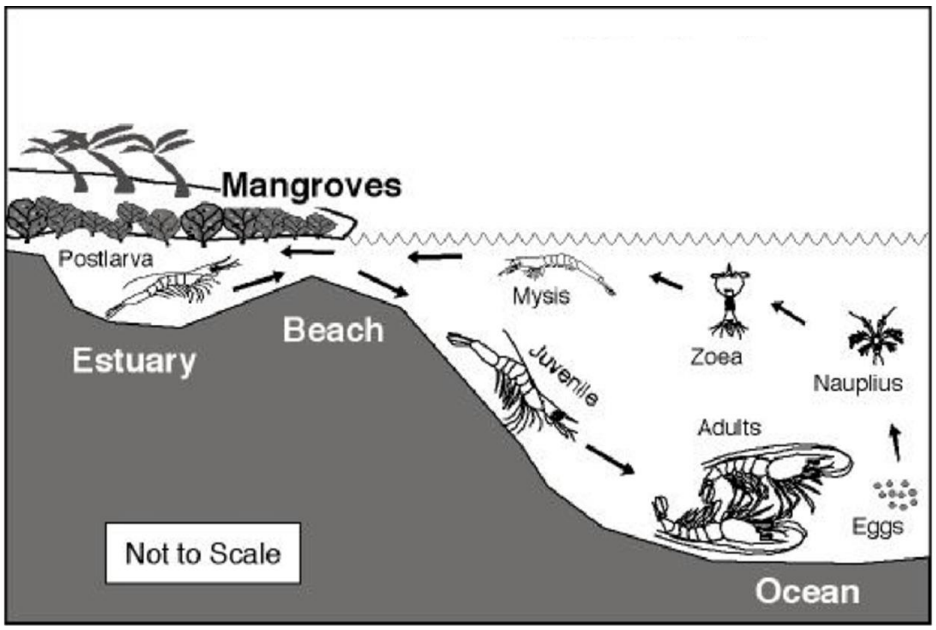


Figure 4: The typical life cycle of marine shrimps

Reference: (Rosenberry 2002).

1.2.3 Black tiger shrimp (*Penaeus monodon*)

Black tiger shrimp is the common name of *Penaeus monodon* (Fabricius 1798) belong to the Arthropoda, the animal kingdom's largest phylum. This group of creatures is distinguished by the presence of paired appendages and a protective cuticle or exoskeleton that surrounds the whole animal. Crustacea is a subphylum of Crustacea that contains 42,000 species, mostly aquatic, divided into ten groups. Shrimp, lobsters, crayfish, and crabs are all members of the Decapoda order, which is part of the Malacostraca class. Black tiger Shrimp is a shrimp that adaptable to the environment, patient, easy to raise and has a good texture. In the past (during 1992-1997), Thailand is considered the number one exporter of black tiger shrimp in the world. Which makes the total export income of not less than 30,000 million baht per year. Therefore, it is popular shrimp and important export product.

1.2.3.1 Morphology

A cephalothorax with a distinctive rough rostrum and a segmented abdomen distinguishes penaeid shrimp on the outside (Figure 5). The cephalothorax houses the majority of the organs, including the digestive system, gills, and heart, while the abdomen houses the muscles. Cephalothorax appendages differ in appearance and function. Sensory functions are performed by antennules and antennae in the head field. The jaw-like structures involved in food absorption are formed by two pairs of maxillae and the mandibles . The maxillipeds are the first three pairs of appendages in the thorax, while the walking legs are the next five pairs, which are specialized for food processing (pereiopods).On the abdomen, there are five pairs of pleopods (swimming legs). (Bailey-Brock and Moss 1992).

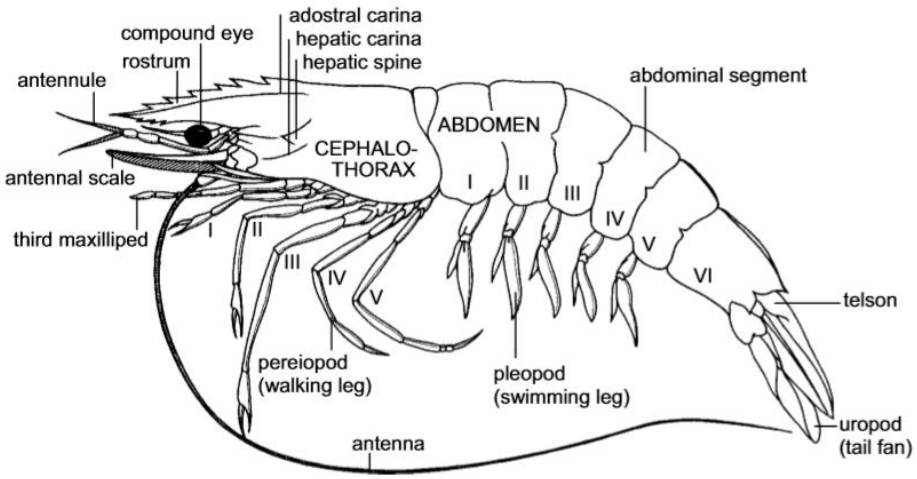


Figure 5: The external morphology of *Penaeus monodon*

Reference: (Primavera 1990).

Figure 6 depicts the internal morphology of penaeid shrimp. The blood and blood cells of penaeids and other arthropods are referred to as haemolymph and haemocytes because they have an open circulatory system. Crustaceans have a muscular heart in the cephalothorax that is located dorsally. The heart's valved haemolymph channels branch numerous times before reaching the sinuses, which are located around the body and serve as a conduit for the exchange of substances. The haemolymph returns to the heart through three deep non-valved openings after passing through the gills. The hepatopancreas occupies a substantial portion of the cephalothorax in penaeid shrimp. Diverticula of the intestine make up this digestive gland. Haemolymph sinuses are spaces between hepatopancreatic tubules. The hepatopancreas key functions include nutrient absorption, lipid storage, and digestive enzyme production (Johnson and Otto 1981). The lymphoid organ, which filters the haemolymph, receives one of the haemolymph veins that leaves the heart. This organ is surrounded by the hepatopancreas, which is positioned ventro-anteriorly. The haematopoietic system produces haemocytes. This organ is located throughout the cephalothorax, although it is most visible around the stomach and at the start

of the maxillipeds. Figure 6 does not display the lymphoid organ or haematopoietic tissue.

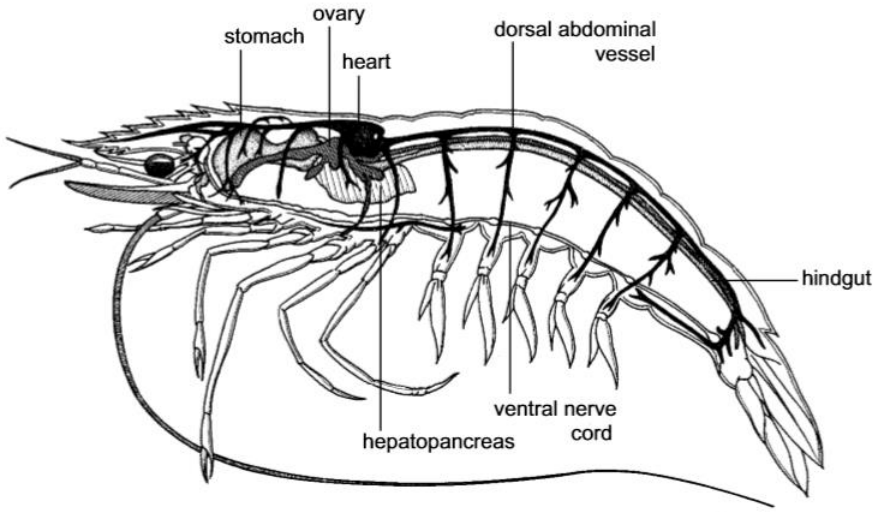


Figure 6: The internal anatomy of a female *Penaeus monodon*

Reference: (Primavera 1990).

1.2.3.2 Distribution and life cycle

The giant black tiger shrimp can be found through most of the Indo Pacific zone, from Japan to Taiwan, east to Tahiti, south to Australia, and west to Africa. The life cycle of penaeid is divided into many phases that can be observed in a number of environments (Figure 7). In their natural habitat, juveniles prefer brackish coastal lines and mangrove estuaries. The majority of the adults move to shallow coastal regions with greater salinity, where they mate and reproduce. Each spawning, females emit between 50,000 and 1,000,000 eggs. The eggs hatch into the nauplius, which is the first larval stage. For a few days, the nauplii feed on their stocks before developing into protozoeae. The protozoeae consume algae before transforming into myses. Myses consume algae and zooplankton, and they retain many characteristics with adult shrimp. They mature into megalopas, also known as postlarvae (PLs). Larval stages

survive in surface waters rich in plankton off the coast and migrate to the coast as they mature. Figure 7 shows the life cycle of the *Penaeus monodon* shrimp. After fertilization, the eggs hatching within 16 hours. Nauplius (6 stages in 2 days), protozoea (3 stages in 5 days), mysis (3 stages in 4-5 days), and megalopa are the larval stages (6-35 days). Megalopa and early juvenile stages are referred known as postlarvae. It takes 135-255 days for a juvenile to mature into a subadult, and 10 months for sexual maturity.

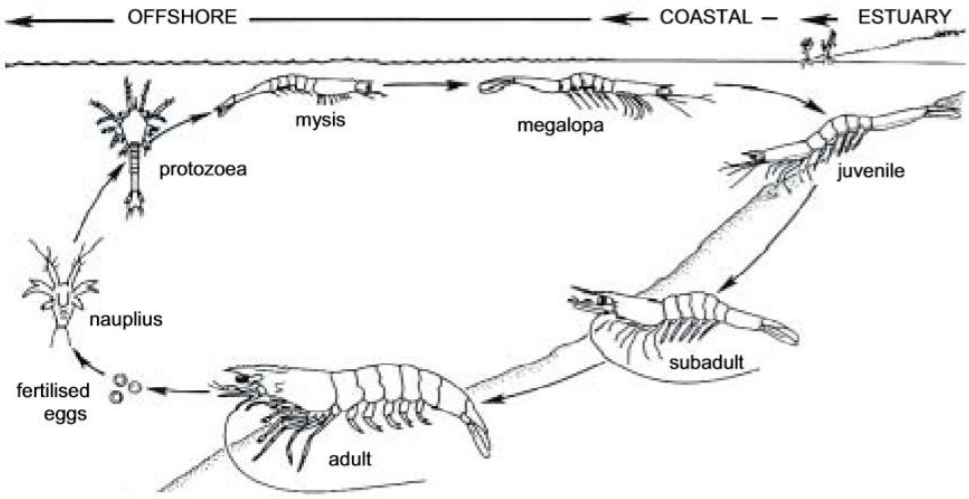


Figure 7: The life history of *Penaeus monodon*

Reference: (Motoh 1984).

1.2.4 Spermatogenesis in shrimp

The first investigation of spermiogenesis and spermatogenesis in decapod crustaceans was carried out in an astacidea, *Procambarus clarkia*, using light and electron microscopes (Moses 1961). For *P. clarkia*, the process of the primordial testis maturing into a fully mature testis was also described in detail (Taketomi et al. 1996). The testis of this crayfish is made up of numerous seminiferous tubules that contain sertoli cells and germ cells at various stages of development. The tubules of seminiferous join together to forming channels for collecting that lead to the vas deferens. Spermatogonia is division during mitosis, then differentiate into primary spermatocytes and secondary spermatocytes, and finally spermatids. After that, spermatids develop into mature sperm, which have a radical arms, acrosome, and a nucleus with strongly decondensed chromatin.

Many species of palaemonid decapods have been studied for their spermatogenesis and sperm structure of the male reproductive system. Poljaroen et al. (2010), they have examined categorized the differentiation of male germ cells stage during spermiogenesis and spermatogenesis in *Macrobrachium resenbergii*, which is the most commonly cultivated freshwater prawn in the world (Chow et al. 1982). It was reported that there are 12 stages in the development of male germ cells, including spermatogonia, primary spermatocyte (6 phase: leptotene, zygotene, pachytene, diplotene, diakinesis and metaphase), secondary spermatocyte, spermatids (3 stages) and mature sperm. The developing germ cells in spermatogenesis contain features that are comparable to those of other invertebrates, such as spermatogonia, that are found in the seminiferous tubules. A spermatogonium is a cell that is oval in form and has a big central nucleus with mainly euchromatin and one or two prominent nucleoli, measuring around 8-10 m in diameter. (Ruiz et al. 2020), they describe the male freshwater prawn *M. carcinus*'s reproductive morphology and spermatogenesis (Figure 8). The male freshwater prawn *M. carcinus*'s reproductive system comprised the lobed of testes that were attached to the vas deferens. *M. carcinus*'s testis was separated into many lobules. Each lobule was generated by surrounding a cluster of germ cells with connective tissue and nurse cells. The acinus wall is made up of thin connective tissue and nurse cells. In the spermatogenic process, germinative cells form on the inside. Primary spermatocytes

of similar size are produced by peripheric spermatogonia. The main spermatocytes give rise to secondary spermatocytes, which are smaller and have a more compact nucleus, at the end of the first meiosis stage. Spermatids are irregularly shaped spermatocytes that form at the end of the secondary spermatocytes meiosis. The spermatozoa are formed when the spermatids are completely polarized. Spermatogenesis occurs in the lobule in the opposite direction of germ cell differentiation.

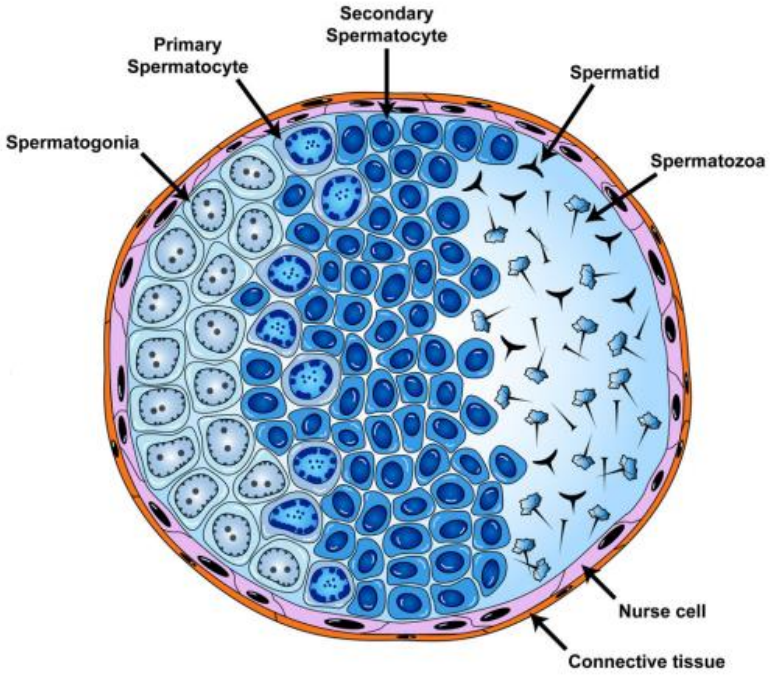


Figure 8: Diagram illustrating the structure of testicular lobule

References: (Ruiz et al. 2020).

The morphology of reproductive in penaeoidean shrimps has been investigated in a lot of species. Fransozo et al (2016), they described about testis and spermatogenesis of the white shrimp *Litopenaeus schmitti* (Burkenroad 1936) (Crustacea, Penaeidea). Each lobule of the testis has a digitiform structure surrounded by connective tissue. Seminiferous tubules form a convoluted net runs through the lobule. Somatic tube filled with spermatozoa and germinal cells can be seen in the seminiferous tubules. The seminiferous duct, which is filled with spike less free spermatozoa, is enclosed by a simple columnar epithelium. The seminiferous tubule's

periphery, generally at the opposite pole of the seminiferous duct, the spermatogonia form a germinal center. Normally, spermatogonia's germinal center is discovered simultaneously with spermatid development. The accessory or Sertoli cells that surround the seminiferous tubules raise their thin cytoplasm during spermiogenesis, becoming eosinophilic and readily apparent. The accessory or The Sertoli cells have many a smooth endoplasmic reticulum and mitochondria and during spermatogenesis. The spermatogonia reveal a big nucleus with at least one nucleoli on transmission electron microscope (TEM). The cytoplasm contains a few of organelles as well as rough endoplasmic reticulum (RER), mitochondria, and tiny electron-dense entities in the perinuclear cytoplasm. The spermatogonia are found on a connective tissue that thin and separates the germinal core from the region where spermatocytes are forming. The nucleus of a primary spermatocyte is larger than that of spermatogonia, and the cytoplasm is slightly larger. Synaptonemal complexes are used to examine and document different stages of meiotic prophase. RER is found in the cytoplasm, and a huge membrane-bound electron-dense vesicle may be observed in some cells. This eosinophilic granule is useful for identifying initial spermatocytes under light microscopy. Spermiogenesis starts in the initial stages spermatid, which features big heterochromatin blocks that grow less condensed as time goes on. Several scattered proacrosomal vesicles mix with one another in the cytoplasm, the cell expands and migrates to the cell periphery. The RER, Golgi bodies, and mitochondria are among the organelles found in the cytoplasm. Large proacrosomal vesicles can be seen on the contrary pole to the nucleus of the mid-spermatid in initial stages. Despite the cytoplasm remains enormous and the nucleus has heterochromatin blocks and RER, the proacrosomal vesicle is referred to as the acrosomal vesicle. The cytoplasm becomes filled with tiny vesicles in the mid-spermatid, and the nucleus has mitochondria with minimal cristae and barely thin chromatin filaments. The spike less late spermatid is discharged into the seminiferous duct. In the spherical main body, the acrosomal cap contains two prominent electron-dense regions that create the potential spike base in longitudinal sections. The acrosomal cap expands inversely on the main body, generating curved lateral extensions. The acrosomal vesicle is generated by the less electron-dense lateral expansion of the acrosomal cap and the spike base in the late spermatid. The granular material beneath the potential spike base differs from the

filamentous chromatin of central and big nuclei. In contrast to the acrosomal, the cytoplasm is constrained to a small area.

1.2.5 Cryopreservation

Cryopreservation is a well-known technique in which liquid nitrogen (-196 °C) is used as a refrigerant to keep biomaterial samples functional for long periods of time (Chao et al. 2009) and it has applications in aquaculture, biotechnology, animal husbandry, and endangered species protection (Golshahi et al. 2018). But cryopreservation of eggs is difficult due to their big size, high lipid and yolk content. Although, the cryopreservation process has been successfully used to preserve spermatogonia cells of fish species such as Nile tilapia (Lacerda et al. 2010), rainbow trout (Lee et al. 2013), and common carp (Franěk et al. 2019) but only recent reported cryopreservation of sperm and spermatophores in shrimp, not yet report mentioned successful cryopreservation of spermatogonia cell.

Cryopreservation involved several conditions that need to increase post-thaw survivability, these conditions including type and concentration of cryoprotectant, equilibration time (Ciereszko et al. 2014), thawing temperature (Nynca et al. 2017), and method for preserved that required for successful cryopreservation.

1.2.5.1 Research of cryopreservation

Cryopreservation of germ cell has been investigated in a variety of animals. Higaki et al. (2018), they used slow-cooling (freezing) and rapid-cooling (vitrification) techniques to examine the possibility of cryopreservation of oogonia and spermatogonia in the highly endangered cyprinid honmoroko (*Gnathopogon caerulescens*). First, they investigated toxicity of the testicular cell and properties of glass-forming in 5 cryoprotectants: dimethyl sulfoxide (DMSO), propylene glycol (PG), ethylene glycol (EG), glycerol (GC), and 1,3-butylene glycol (BG), and estimated cryoprotectant concentrations appropriate for freezing and vitrification solutions, respectively. Then, freezing solutions of

EG, GC, DMSO, PG, and BG at 3, 2, 3, 2, and 2 M, and vitrification solutions at 7, 6, 5, 5, and 4 M were prepared, respectively. The testicular cells, which mostly contained spermatogenic cells in their early stages (such as primary spermatocytes and spermatogonia), were cryopreserved and cultured for seven days before being immunochemically tagged against the germ cell marker protein Vasa. Vasa-positive cell-infested areas were suggested that the vitrification resulted in higher germ cell survival than freezing, and the best performance was achieved with 5 M PG, which resulted in roughly 50% germ cell recovery after vitrification. The cells were cryopreserved by vitrification with 5 M DMSO resulted in the greatest oogonia survival in ovarian cells including oogonia and stage I, II, and IIIa oocytes, with cell numbers comparable to those cultured without vitrification. The current findings imply that adequate cryoprotectants for spermatogonia and oogonia can be used to effectively cryopreserve gonial cells of male and female endangered species *G. caerulescens.*

Abualreesh et al. (2020), they discovered the best permeating (DMSO, EG, glycerol, and methanol) and non-permeating (trehalose or lactose with BSA or egg yolk) cryoprotectants, their optimal concentrations of cryoprotectants, and the best freezing rates at −0.5, −1.0, −5.0, −10 °C/min till −80 °C that yield the most viable type A spermatogonia cells. All parameters had a significant impact on formation and viability of cell after thawing, according to the findings. At 1.0 M concentration, DMSO was the most efficient penetrating cryoprotectant. Because of interactions between the two components, the efficient concentration in each cryoprotectant differed depending on the specification of cryoprotectant. In comparison to the 1.0 M DMSO control, 0.2 M lactose with egg yolk consistently improved type A spermatogonia production and viability among the non-permeating cryoprotectants. 1 °C/min was the best overall freezing rate, however, 0.5 °C/min had similar results. To achieve the greatest cryopreservation results, they recommend freezing testicular tissues of blue catfish in DMSO at 1.0 M concentration with lactose at 0.2 M concentration and egg yolk at a rate of -0.5 or 1 °C/min.

Although the cryopreservation method has been used to successfully preserve fish's spermatogonia cells, but cryopreservation in shrimp not yet report mentioned successfully preserve spermatogonia cell. The cryopreservation in shrimp has been used to successfully preserve sperm such as: Vuthiphandchai et al (2007), they determined the effect of cryoprotectants on sperm viability and developed a freezing method for long period storage spermatophores of *P. monodon*. Spermatophores were suspended for 30 minutes in calcium-free saline (Ca-F saline) containing the cryoprotectants: DMSO, EG, PG, formamide, and methanol at 5, 10, or 20% concentrations were examined using a modified eosin–nigrosine staining technique. Because DMSO caused the least losses in apparent sperm viability, a freezing process utilizing Ca-F saline including 5% DMSO was designed. Three protocols were used to cryopreserve spermatophores: cooling to a final temperature (30, 80, or 80 °C) and then rapidly freezing to liquid nitrogen at 2, 4, 6, 8, 10, 12, 14, or 16 °C/min colling rated. After cryopreservation, spermatophores were thawed at 30, 60, 70, or 90 °C for 2 minutes. To successfully cryopreserve spermatophores in liquid nitrogen used cool at a rate of 2 °C/min between 25 and 80 °C in one step. After cryopreserving at 210 days, spermatophores were thawed for 2 minutes in a 30 °C water bath and provided viable sperm. Average of sperm viability in fresh spermatophores (97.8 ± 2.9%) and cryopreserved spermatophores preserved for less than 60 days (87.3 ± 4.1%) did not difference ($P > 0.05$); however, spermatophores were cryopreserved between 90 to 210 days were lower ($P < 0.05$) and ranged from 27.3 ± 3.4 to 53.3 ± 4.3%. Fertilized eggs were produced using thawed spermatophores that had been stored in liquid nitrogen for fewer than 62 days(fertilization rates of 71.6–72.2% and hatching rates of 63.6–64.1% and fresh spermatophores rates of 70.8–78.2% and 66.3–67.8%, respectively). Finally, cryopreserved spermatophores were stored in liquid nitrogen for up to 210 days, sperm within cryopreserved spermatophores maintained their viability.

Castelo-branco et al. (2015), they used soy lecithin as an extracellular cryoprotectant for *Litopenaeus vannamei* sperm cryopreservation by using the vitrification technique. Three intracellular cryoprotectants (DMSO, EG, and MeOH at final concentrations of 5, 10, and 30% (v/v)) in sterile calcium-free saline solution (Ca-F) were evaluated in the toxicity assay. After extrusion, the sperm masses were immersed for 10, 30, and 120 min in 0.5 ml of cryoprotectant solutions. Then apparent sperm viability (ASV) was determined by staining with eosin–nigrosine. Methanol was chosen because it had a minimal decrease of ASV (21%) at 30% concentration and 120 min exposure duration. In vitrification procedure, soy lecithin was tested as an extracellular cryoprotectant, whether or not it was combined with methanol. For vitrification, sperm masses were submerged in cryoprotectant solutions follow: (1) CaF + 0.4 M trehalose as the base solution, (2) base solution + 30% MeOH, (3) base solution + 30% MeOH + 1% soy lecithin (4) base solution + 30 % MeOH + 2% soy lecithin (5) base solution + 1% soy lecithin and (6) base solution + 2% soy lecithin. Then, the samples were stored in liquid nitrogen by using vitrification procedure at 1, 30, 60, and 120 days and evaluated with fluorescence microscopy. Trehalose was supplemented with soy lecithin that is an excellent extracellular cryoprotectant by using vitrification procedure, it remained high and consistent for all studied solutions over the period at 120 days (88.6%). The vitrification procedure found to be effective for *L. vannamei* sperm mass cryopreservation with high rates of sperm membrane integrity. This appears to be the first report for cryopreservation of penaeid sperm mass by using the vitrification method.

Memon et al. (2012), they investigated the effects of several cryoprotectants on sperm viability and optimized the cryopreservation procedure for spermatophores for long-term storage of Banana shrimp (*Penaeus merguiensis*). Spermatophores were suspended in Ca-F saline for 15 min, then thawed at 27°C using Ca-F saline with $MgCl_2$ at 15 % concentration and equilibrated at room temperature (25°C) for 15 minutes. Exposure and cooling rate at 25, 20, 16, 4, 2, -4, -20, -80, -150°C/10 min were selected. A modified

eosin-nigrosine staining technique was employed to examine sperm viability. Although $MgCl_2$ caused the smallest losses in apparent sperm viability, the freezing process was devised using Ca-F saline with $MgCl_2$ at 15 % concentration. Spermatophores were stored for 180 days at -196°C in liquid nitrogen utilizing the above exposure/cooling rate. Sperm viability of fresh spermatophore (93.8±1.3%) and cryopreserved spermatophore retained at 24 h and 60 days (83.5±0.6 and 61±1.2) had similar sperm viability ($p>0.05$), however for spermatophore preserved in liquid nitrogen at 90 to 180 days had reduced ($p<0.05$) (55.4±0.3 – 16.4±1.2). Previously, the spermatophore was stored in liquid nitrogen at 60 to 90 days, sperm vitality was decreased significantly ($p<0.05$) after storage at 90 days. The viability of spermatophore preserved at 120 and 150 days were 48.9±0.9 and 32.4±0.9%, respectively and the viability of spermatophores preserved at 150 and180 days was low (<35%). *F. merguiensis* females were artificially inseminated with cryopreserved spermatophore that maintained in liquid nitrogen for 7 to 30 days and 60 to 90 days had fertilization rates of 73.9±1.5 to 66.7±3.1 and 67.3±3 to 64.1±2.1%, respectively but fresh spermatophore had fertilization rates of 88.2±1.5%. Eggs were fertilized with cryopreserved spermatophore that retained in liquid nitrogen for 7 to 30 days and for 60 to 90 days had hatching rates of 77.6±2.5 to 72.2±3.5 and 81.5±12.1 to 62.5±1.5, respectively which were not significantly ($p>0.05$) from the control group (76.2±13.5%). In summary, sperm viability in cryopreserved spermatophore that maintained in liquid nitrogen for 90 days was high, but sperm viability in cryopreserved spermatophore that maintained in liquid nitrogen for 180 days was decreased.

1.2.5.2 Freezing process

There are two cryopreservation techniques that are often utilized: slow-freezing and vitrification.

1.2.5.2.1 Slow freezing

The slow cooling cryopreservation, which is the most used technique worldwide (Gurruchaga et al. 2018) and has been the conventional technique. Slow-freezing permits cryopreservation to take place at a slow sufficient rate to allow for appropriate cellular dehydration while limiting intracellular ice accumulation. (Rienzi et al. 2017) and cryoprotectants (CPAs) permeability, which refers to the diffusion effect of CPAs across cell membranes, protects cell natures (Zhang et al. 2018).

Lee and Yoshizaki (2016), they successfully cryopreservation of spermatogonia. Whole testes of dangerously endangered Manchurian trout (*Brachymystax lenok*) were cryopreserved in cryomedium containing 1.3 M of methanol, 0.2M of trehalose and 10% of egg yolk at -1^{o}C/min cooling rate, then stored for 2 days in liquid nitrogen and thawed in a water bath at 30^{o}C.

1.2.5.2.2 Vitrification

Vitrification is a modern cryopreservation method that combines an incredibly fast cooling rate for cells with an extremely high concentration of CPAs in the cell suspension, which will lower the freezing temperature before rapid cooling (Guven and Demirci 2012). Cell culture media containing a high concentration of CPAs underwent a rapid transformation from liquid to glass during the vitrification phase, minimizing cell harm (Zhang et al. 2018). Vitrification reduces the risk of mechanical damage to cells by preventing the development of ice crystals. Vitrification, in comparison to slow freezing, has the benefits of being a time saving, quick process that requires no special or costly equipment. Vitrification has also been shown to maintain the morphologic integrity of stromal cells better than slow freezing. (Shi et al. 2017).

Vitrification appears to be a burgeoning alternative to slow freezing, as it has proven to be successful. Seki et al. (2017) applied for cryopreservation for whole testes of medaka. Whole testes of medaka on a copper mesh were directly immersed to liquid nitrogen after 20 minutes of exposure in vitrification solution containing 30% (v/v) EG, 21% (w/v) ficoll, and 0.35M sucrose at 0°C, warmed by direct immersion in 0.2M sucrose at 25°C, and whole testes were submerged to L-15 medium. Therefore, they have been cryopreserved effectively for spermatogonia cells and have a high viability.

Both approaches have disadvantages for cryo-injured cells during the cryopreservation process, such as ice injury (Zhurova et al. 2010) from cooling phase ice crystal formation, osmotic injury from loading and unloading CPAs, and CPAs toxicity. Both cryopreservation methods can result in a loss of cell viability and function due to the permeability and concentration of CPAs (Zhang et al. 2018). Therefore, identifying an optimal type and concentration of cryoprotectant is necessary for a long-term preservation of spermatogonia cells of shrimp.

1.2.5.3 Cryoprotectants (CPAs)

Cryoprotectants are used to prevent or minimize cold shock damage, intracellular ice formation, thawing recrystallization, and membrane instability. By the amount of liquid fraction in the sample, cryoprotectants will lower the sample's freezing point and the amount of solutes and salts, as well as preventing the development of ice both outside and inside cells (Royere et al. 1996). While the oxygen atoms in molecules of water bind with hydrogen bonds between membrane phospholipids in biological structures, Water replaces cryoprotecting molecules such as glycerol. The integrity of the membrane is dependent on these bonds (Crowe et al. 1984). A successful the cryoprotectant should be soluble in water and have a low toxicity level (Alvarenga et al. 2005). Cryoprotectants

may be classified as penetrating or non-penetrating cryoprotectants based on their mechanism of action (Figure 9).

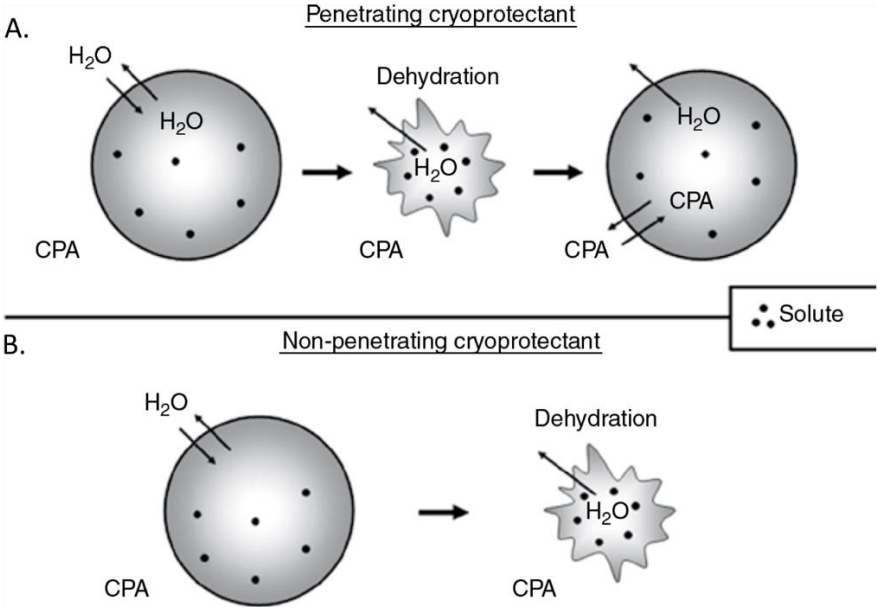


Figure 9: Groups of cryoprotectants (CPAs): A. Penetrating cryoprotectants and B. Non-penetrating cryoprotectants

Reference: (Chian and Quinn 2010).

1.2.5.3.1 Penetrating cryoprotectants

Penetrating cryoprotectants which have a low of molecular weight allows them to penetrate cells. They can influence both the extracellular and intracellular environments in this way.

Because of the chemical potential of water differential between the outside and inside of cells, cytosolic water travels to the cell's outer milieu when cryoprotectant is added to the cell suspension. Due to the concentration variation, the penetrating cryoprotectant penetrates cells. This process continues until the extracellular and intracellular conditions

for both cryoprotectant and water have established equilibrium. Intracellular ice formation (IIF) may be avoided or avoided by lowering the intracellular medium 's freezing point. Penetrating cryoprotectants enter cells, breaking hydrogen bonds between water molecules to create new hydrogen bonds and changing the water structure as a result. The cryoprotective feature is demonstrated in this way by avoiding the cells from accumulating high ion concentrations and avoiding significant dehydration as a result of water loss during freezing (Sagirkaya et al. 2003).

Glycerol, DMSO, and PG are penetrating cryoprotectants which change the physical characteristics of intracellular solution, decrease intracellular ice formation, increase resistance to cold shock, regulate the protein and lipid structure of the cell membrane, and increase membrane fluidity to influence spermatozoa during cryopreservation (Purdy 2006).

Glycerol (GLY)

Three hydroxyl groups are found in a glycerol molecule. Each glycerol molecule has the ability to bind to three molecules of water. Glycerol can quickly move through the membrane pores due to its smaller molecular size. The hydroxyl bonds produced between water molecules strengthen and solidify as the temperature decreases. As a result, the frozen water increases and causes cell damage. Glycerol functions as a cryoprotectant by attaching with the hydrogen atoms of water molecules and interlocking them during freezing to prevent large-volume ice crystals.

Dimethyl sulfoxide (DMSO)

At room temperature, DMSO is a liquid that ranges in hue from colorless to yellow. Chloroform, acetone, ethanol, diethyl ether, benzene, and aromatic compounds are all soluble in it. DMSO has bacteriostatic, anti-inflammatory, diuretic, tranquilizing, and local analgesic properties in addition to being a cryoprotectant for sperm. It's both a penetrant and a messenger, and it can boost insulin's effectiveness (Pope and Oliver 1966).

Ethylene glycol (EG)

When ethylene glycol reacts with water, it changes the hydrogen bonding. Freezing point of purified ethylene glycol has around 12°C and the freezing point drop when mixed with 40% water and 60% ethylene glycol, making it incapable of forming crystalline structures. Ethylene glycol is the most effective choice for cryoprotection because of its capability. However, ethylene glycol has been linked to gastrointestinal discomfort, pulmonary edema, and lung inflammation (Bhattacharya 2018).

Propylene glycol (PG)

Propylene glycol or propane-1, 2-diol is a molecule of synthetic organic with the chemical formula $C_3H_8O_2$ that is non-irritating. It has a mildly sweet flavor and is colorless and odorless. It is a diol chemically, and it is miscible with water, chloroform, and acetone. It is a common deicing fluid for planes. RV or marine antifreeze are both brands of propylene glycol. It also has the antifreeze property of an automobile (Bhattacharya 2018).

1.2.5.3.2 Non-penetrating cryoprotectants

Non-penetrating cryoprotectants are cryoprotectants that do not enter the sperm membrane and solely work in the extracellular environment. They often work by integrating the structure of membrane or lowering the medium's freezing point (Purdy 2006). The two categories of cryoprotectants that are typically divided are high molecular weight cryoprotectants and low molecular weight cryoprotectants. (Woelders 1997). Non-penetrating cryoprotectants with low molecular weight include monosaccharides: glucose and galactose, disaccharides: sucrose and trehalose, and trisaccharides: raffinose and melezitose. Cryoprotectants with a high molecular weight such as ficoll 70, polyvinyl alcohol (PVA), polyethylene glycol (PEG), polyvinyl pyrrolidone (PVPP), polyethylene oxide (PEO), and others work by lowering the number of ice crystals that form on the outside of cells during the freezing/thawing process, thereby reducing cellular damage.

Sugars are used as an energy source, a cryoprotectant, an osmotic pressure regulator, and a reducer ice formation of cellular during sperm cryopreservation (Bucak and Tekin 2007). Trehalose which is a disaccharide produced by joining two D-glucose molecules and increases the distance of membrane phospholipids by attaching to the polar portion of the cell membrane’s phospholipids These cavities in the membrane enable the outflow of water from the cell freezing, preventing the harmful effects from cellular dehydration, preventing the formation of ice in the cell and stabilizing the cell membrane.

Cooling sperm consumes energy from the environment, Van der Waals forces cause phospholipids to accumulate, and the liquid crystal phase changes to the gel phase. In the cell membrane during thawing, irregular voids form. This results in membrane disruption, leakage of ions and water into and out of the cell, as well as cell death (Patist and Zoerb 2005). As trehalose is applied to the medium which generating gaps in the cell membrane between phospholipids, preventing them from

aggregating during freezing and protecting the membrane structure's integrity after thawing (Ahmad et al. 2012).

1.3 Objectives of Research

1.3.1 To identify the germ cells developmental stage for cryopreservation.

1.3.2 To optimize the equilibration time for slow freezing method, type, and concentration of cryoprotectants, and thawing temperature for preserve spermatogonia cell of shrimps including *Marcrobrachium Rosenbergii*, *Fenneropenaeus merguiensis and Penaeus monodon*.

1.3.3 To compare the efficiency of slow freezing and vitrification methods for a long-term preservation of spermatogonia cells.

CHAPTER 2

RESEARCH METHODOLOGY

2.1 Materials

2.1.1 Chemicals

2.1.1.1 Analytical grade

Chemicals	Company
3-Aminopropyltriethoxysilane	Sigma
5-bromo-4-chloro-3-indolyl phosphate (BCIP)	Roche
Acetic acid	RCI Labscan
Acetic anhydride	RCI Labscan
Agarose gel	Vivantis
Antibiotic-antimycotic	Invitrogen
Blocking reagent	Roche
Bovine serum albumin (BSA)	Sigma
Calcium chloride dehydrate ($CaCl_2 \cdot 2H_2O$)	LOBA chemi
Chloroform	RCI Labscan
Citric acid dihydrate	RCI Labscan
Collagenase H	Roche
Dextran sulfate	Sigma
Diethyl Pyrocarbonate (DEPC)	Bio basic
Dimethyl sulfoxide (DMSO)	RCI Labscan
Di sodium hydrogen phosphate (Na_2HPO_4)	LOBA chemi

2.1.1.1 Analytical grade (Continue)

Chemicals	Company
Dispase II	Sigma
DNAse I	Sigma
Entellan neu	Merck
Eosin	Leica
Ethanol	RCI Labscan
Ethylenediaminetetraacetic acid (EDTA)	LOBA chemi
Ethylene glycol	RCI Labscan
Fetal Bovine Serum (FBS)	Gibco
Formalin	RCI Labscan
Formamide	LOBA chemi
Glacial acetic acid	Lab scan
Glucose	RCI Labscan
Glycerol	Ajax chemical
Guava Nexin® Reagent	Merck
Hematoxylin	Leica
HistoVT one	NACALAI Tesque
Hydrogen chloride (HCL)	RCI Labscan
Hydroxyethyl piperazineethanesulfonic acid (HEPES)	Promega
Isopropanol	RCI Labscan
Magnesium chloride Hexahydrate	LOBA chemi
Leibovitz's L-15 powder medium	Life technologies
Maleic acid	RCI Labscan
Nitroblue tetrazolium (NBT)	Roche
Paraformaldehyde	RCI Labscan
Picric acid	RCI Labscan
Potassium acetate	RCI Labscan

2.1.1.1 Analytical grade (Continue)

Chemicals	Company
Potassium chloride	LOBA chemi
Potassium hydrogen phosphate (KH_2PO_4)	LOBA chemi
Proteinase K	Invitrogen
Sodium citrate	RCI Labscan
Sodium chloride	RCI Labscan
Sodium dodecyl sulfate (SDS)	Himedia
Sodium hydrogen phosphate (Na_2HPO_4)	RCI Labscan
Sodium hydroxide (NaOH)	RCI Labscan
Sodium pyruvate	Sigma
Sucrose	RCI Labscan
Trehalose	New Jersey
Tris-base	Himedia
Tris buffer	LOBA chemi
Tri-sodium citrate dihydrate	RCI Labscan
TRIzol® reagent	Gibco
Trypan blue	Gibco
Tryptone	Himedia
Tween 20	LOBA chemi
Yeast extract	Himedia

2.1.1.2 Molecular biology grade

Chemicals	Company
100 bp DNA Ladder	Promega
10x *EcoR I* buffer	Promega
10x Ligation buffer	Thermo Scientific
Alexa Fluor 488	Invitrogen
Ampicillin	Biobasic
AMV reverse transcriptase	Promega
Apa I enzyme	Biolab
CutSmart buffer	Biolab
Deoxynucleoside triphosphate (dNTP)	TaKaRa
EcoRI enzyme	Promega
Go taq® green master mix	Promega
Heparin	Himedia
Polyclonal rabbit anti-vasa antibody	Abcam
Random primer	Promega
RNase inhibiter	Roche
Spe I enzyme	Biolab
Taq DNA polymerase Prime SATAR	TaKaRa
Taq DNA polymerase buffer Prime SATAR	TaKaRa
T4 DNA Ligase	Thermo Scientific
Xylene	RCI Labscan
Yeast tRNA	Invitrogen

2.1.2 Bacteria

Escherichia coli (Top 10 F') Genotype: F' { laclq Tn10(TetR0 } mcrA Δ (mrr-hsdRMS-mcrBC) Φ80lacZΔM15 ΔlacX74 recA1 araD139 Δ(ara-leu)7697 galU galK rpsL (StrR) endA1 nupG, Invitrogen, Netherlands.

2.1.3 DNA vector

pGEM®-T Easy vector (Promega, Madison, USA)

2.1.4 Oligonucleotide primer

Primers	Sequence (5' → 3')
Forward *Vasa*-741	5'-TCC AGG GGA GGT GGT AGA GG-3'
Reverse *Vasa*-741	5'-ACT CCC TGT CTG AGC ACA GC-3'
Forward *Vasa*-846	5'- TCC AGG GGA GGT GGT AGA GG -3'
Reverse *Vasa*-846	5'-GAT CAC CAG TCC CGT TGG TT-3'
Forward *β* -actin	5'-GCT ACA GCT TCA CCA CCA CCG-3'
Reverse *β* -actin	5'-GAT GTC CAC GTC RCA CTT CAT-3'

2.1.5 Animals

2.1.5.1 Giant freshwater prawn

Giant freshwater prawn, *Marcrobrachium Rosenbergii* (n = 62) with mean body weight of 36.15 ± 8.78 g and total body length of 16.04 ± 1.40 cm were obtained from Songkhla lake in Songkhla province, Thailand. Male giant freshwater prawns were maintained to cement pond (1.5 x 2 x 0.5) containing half tap water at 27-28 °C for 12 h light and 12 h dark and they were fed twice

a day with commercial feed by dividing into two equal portions for the morning and evening feeding. Shrimp were held for 5-7 days before the beginning of the experiment and water were exchanged daily at approximately 50% until finished in the experiment.

2.1.5.1 Banana shrimp

Banana shrimp, *Fenneropenaeus merguiensis* (n = 68) with mean body weight of 11.74 ± 2.36 g and total body length of 12.25 ± 0.89 cm were obtained from Trang Coastal Fisheries Research and Development Centre, Trang province, Thailand. All males were maintained in cement pond (1.5 x 2 x 0.5) containing sea water (30 ppt) at 27-28 °C for 12 h light and 12 h dark and they were fed twice a day with commercial feed by dividing into two equal portions for the morning and evening feeding. Shrimp were held for 5-7 days before the beginning of the experiment and water were exchanged daily at approximately 50% until finished in the experiment.

2.1.5.3 Black tiger shrimp

Black tiger shrimp, *Penaeus monodon* (n = 66) with mean body weight of 12.62 ± 1.68 g and total body length of 12.29 ± 0.97 cm were obtained from Chana district, Songkhla province, Thailand. All males were maintained in cement pond (1.5 x 2 x 0.5) containing sea water (30 ppt) at 27-28 °C for 12 h light and 12 h dark and they were fed twice a day with commercial feed by dividing into two equal portions for the morning and evening feeding. Shrimp were held for 5-7 days before the beginning of the experiment and water were exchanged daily at approximately 50% until finished in the experiment.

2.2 Equipment

0.45 nylon µm-pore nylon filter
24 well plate
48 well plate
Analytical balance 4 digits
Autoclave
Automatic pipette
Automatic tissue processor
Biosafety cabinet class II
Centrifuge
Copper mesh (clever, Aichi, Japan)
Cryovials (NEST)
Embedding Center
Flow cytometer
Fluorescence microscope
Freezer container
Freezer -20 °C
Freezer -80 °C
Gel documentation
Gel Electrophoresis machine
Heat box
Hematocytometer
Hot air oven
Hybridization oven
Incubator
Incubator shaker
Laboratory Chemical Fume Hood
Light microscope
Liquid Nitrogen container
Magnetic stirrers

Microcentrifuge tube

Microtome

Nano drop

PCR thermal cycler

PCR tube

pH miter

Polymerase Chain Reaction (PCR) machine

Refrigerated Centrifugation

Shaker

Surgical instruments

Water bath

2.3 Methods

2.3.1 Samples collection

The males of shrimp were euthanized by immersion on ice then weighed and measured the length. The various organs including muscle, gill, hepatopancreas, heart, intestine, thoracic ganglion, lymphoid, and testes were aseptically removed and excised from adult shrimp. For tissue distribution analysis, all of tissues were store in TRIzol® reagent (Ambion® by life technologies, USA) at -80 ^{o}C until RNA extraction. For histological analysis, whole testes were fixed into Bouin's solution (1% Acetic acid , 24% Formalin and 75% Picric acid, which is a fixative solution) at 4 ^{o}C for 7 h then transferred into 70% ethanol until histology and for cryopreservation experiments, whole testes were transferred into Leibovitz's L-15 medium (pH 7.8) (Gibco®, USA) containing 27.75 mM D-Glucose (RCI Labscan), 10% Fetal Bovine serum (FBS) (Gibco®, USA) and 1% antibiotic–antimycotic (Invitrogen) before used. All experiments were carried out according with the Guidelines for the Care and Use of Laboratory Animals, Prince of Songkla University, Songkhla, Thailand.

2.3.2 Immunohistochemical analysis

For identification of germ cells developmental stages in testes gonads of the giant freshwater prawn. The whole testes of shrimp were fixed in Bouin's solution fixative at 4 °C for 7 h, dehydrated in ethanol series, embedded in paraffin, and then the tissue blocks were sectioned into 5 µm serial sections. After deparaffinization, some slides were stained with hematoxylin and eosin (H&E) (Leica, Leica Biosystems Richmond, Inc., USA). For immunohistochemical analysis, adjacent deparaffinized slides were immunohistochemically stained with the protein Vasa, which is a marker for germ cells (Raz 2000), by deparaffinized slides were subjected to antigen retrieval using HistoVT One (pH

7.0; NACALAI Tesque, INC. Kyoto, Japan) for 20 min at 90 °C. After incubation, the slides were moved to distilled water (DW) immediately and 2 min washed for three times, and once in 1x Phosphate-buffered saline containing 0.1% Tween 20 (PBST) for 2 min with shaking. Slides were then incubated in blocking reagent (Block Ace powder, KAC Co. Ltd., Japan) in the moisturizing box for 1 h at room temperature with shaking. Then, slides were mounted with polyclonal rabbit anti-vasa antibody (ab2099710, Lot:GR3256982-6, Abcam; Diluted to 50 times with PBS (-)) in immunostain Solution A; diluted 1:200 (Can Get Signal®, Code: NKB-501, TOYOBO) at 4 °C overnight. Subsequently, slides were incubated with diluted Alexa Fluor 488-conjugated goat anti rabbit IgG (Invitrogen, US) in immunostain Solution B; diluted 1:200 (Can Get Signal®, Code: NKB-601, TOYOBO) for 1 h at room temperature in the dark area. Images were obtained using fluorescence microscope (Olympus-IX 70, Olympus, Tokyo, Japan), and germ cell stages were identified according to the classification reported in the giant freshwater prawn (Poljaroen et al. 2010).

2.3.3 Tissue distribution analysis by RT-PCR

Various tissues (testes (T), muscle (MU), gill (G), hepatopancreas (HP), heart (H), intestine (IN), brain (BR), thoracic ganglion (TG) and lymphoid (LY)) from adult of banana shrimp and black tiger shrimp were homogenized and utilized for total RNA extraction with TRIzol® reagent, the tissues were homogenized in 1.5 ml tube with TRIzol® reagent using homogenizer and placed at 15-30 °C for 5 min. Then, the tissues were added chloroform, mixed, placed at 15-30 °C for 2-3 min, centrifuged for at 12,000 rpm for 15 min at 4 °C and removed the supernatant to new 1.5 ml tube. The supernatant was added isopropanol, placed at 15-30 °C for 10 min, centrifuged for at 12,000 rpm for 10 min at 4 °C and discard the supernatant. Total RNA precipitate forms a white gel-like pellet at the bottom of the tube. The pellet was washed with cold absolute ethanol, centrifuged for at 12,000 rpm for 5 min at 4 °C and discard

the supernatant. The pellet was dried at room temperature for 30 min and resuspended in RNase-free water. The quality of the isolated RNA was then assessed using nano drop spectrophotometers (Thermo Fisher SCIENTIFIC). Two micrograms of purified total RNA were reverse transcribed to cDNA using AMV Reverse Transcriptase (Promega, Madison, USA), two micrograms of purified total RNA and the random primers (Promega, Madison, USA) were mixed in 1.5 ml tube, incubated in heat box at 70 °C for 5 min and incubate on ice for 5 min immediately. 10 mM dNTP, 5x RT buffer, AMV and RNase free water were added to 1.5 ml tube in a total volume 25 µl and incubated at 48 °C for 2 h. The cDNA was used for PCR.

For banana shrimp, the PCR reaction were carried out using 1000 ng of cDNA, 0.625 mM of dNTP, 0.25 µM of each primer, 5x Prime STAR buffer (Mg^{2+}plus) and 2.5 units of Prime STAR HS DNA polymerase (Takara, Japan) in a total volume of 12.5 µl, performed at 94°C for 5 min; followed by 35 cycles of 30 s at 94°C, 30 s at 65°C, and 30 s at 68°C; followed by a final elongation step of 68°C for 10 min, with Vasa-specific primers (Vasa_F: 5'-TCC AGG GGA GGT GGT AGA GG-3', Vasa_R: 5'-ACT CCC TGT CTG AGC ACA GC-3') that were designed from the highly conserved regions of *vasa* homologs in shrimp species with the following GenBank accession numbers; *Litopenaeus vannamei*: DQ095772.2 and *Fenneropenaeus merguiensis*: MZ173499 and β -actin-specific primers (β -actin _F: 5'-GCT ACA GCT TCA CCA CCA CCG-3', β -actin _R: 5'-GAT GTC CAC GTC RCA CTT CAT-3') as an internal control designed to amplify the 741- and 300-bp amplicons, respectively. Then, PCR products were detected using 1.5% agarose gel electrophoresis.

For black tiger shrimp, the PCR reaction were carried out using 1000 ng of cDNA, Go taq® green master mix, 2x (Promega, Madison, USA) and 1 µM of each primer in a total volume 12.5 µl, performed at 94°C for 5 min; followed by 35 cycles of 1 min at 94°C, 1 min at 65°C, and 1 min at 72°C; followed by a final elongation step of 72°C for 7 min, with Vasa-specific primers (Vasa_F: 5'-TCC AGG GGA GGT GGT AGA GG -3', Vasa_R: 5'-GAT CAC CAG TCC CGT TGG TT-3') that were designed from the highly conserved regions of *vasa*

homologs in shrimp species with the following GenBank accession numbers; *Litopenaeus vannamei*: DQ095772.2 and *Fenneropenaeus merguiensis*: MZ173499 and β-actin-specific primers (β-actin _F: 5'-GCT ACA GCT TCA CCA CCA CCG-3', β-actin _R: 5'-GAT GTC CAC GTC RCA CTT CAT-3') as an internal control designed to amplify the 846-bp and 300-bp amplicons, respectively. Then, PCR products were detected using 1.5% agarose gel electrophoresis and showed the predicted molecular weight.

2.3.4 The Vasa Probes synthesis

Total RNA was isolated from testes of adult banana shrimp and black tiger shrimp males using the TRIzol® reagent according to manufacturer's instructions. Two micrograms of total RNA were used for cDNA synthesis. First-strand cDNA were synthesized using AMV Reverse Transcriptase and the PCR reaction of Vasa were performed to the method describe in 2.3.3. After that PCR products were separated on a 1.5% agarose gel, the cDNA fragments that showed the predicted molecular weight were isolated and purified using QIAquick® PCR Purification Kit (QIAGEN, Hilden, Germany). The purified cDNA fragments were cloned into the pGEM®-T Easy vector (Promega, Madison, USA). The ligation of purified cDNA fragments with pGEM®-T Easy vector were carried out using vector DNA, insert DNA, 10x buffer, T4 DNA ligase and DI water in a total volume 5 µl and incubate at room temperature for 3 h. Then, the ligated cDNA fragments were transformed to competant cell, taped, incubated on ice for 30 min, heat shock for 90 s, incubated on ice for 5 min immediately, added LB broth and shaked at 180 rpm at 37 °C for 2 h. After that, transformed cDNA were spreaded on LB agar with ampicillin plate and incubated at 37 °C for 16-18 h. The colony were picked and cultured in LB broth at 37 °C for 16 h. Bacteria culture were centrifuged at 12,000 rpm for 1 min, discard the supernatant and remove any media. The cell pellet was completely resuspended in solution I by vortexing, incubated at room

temperature for 5 min, added solution II and mixed by inverting; the cell suspension should be clear immediately and incubated on ice for 5 min. Protein were precipitated with solution III by inverting and incubated on ice for 30 min. The cell suspension was centrifuged at 12,000 rpm at 4 °C for 15 min. A compact white pellet will from along the side or at the bottom of the tube, carefully removed all of the clear lysate to new tube. DNA were precipitated with isopropanol by inverting, incubated at -20 °C at least 30 min, centrifuged at 12,000 rpm at 4 °C for 15 min and discarded the supernatant. The pellet of DNA was washed with 70% ethanol, centrifuged at 12,000 rpm at 4 °C for 15 min and discarded the supernatant. The pellet DNA were dried at room temperature for 30 min and resuspended with DI water. The cDNA was detected using 1% agarose gel electrophoresis and using pGEM®-T Easy vector as a control. Inserted cDNA fragments were sequenced (1st BASE, Inc., Singapore). Then, the sequences were confirmed using the BLAST program.

For creating Vasa probes, the plasmid clones were purified with Plasmid Miniprep Plus Purification Kit (GMbiolab Co., Ltd., Taiwan). Then, 3-5 µg of the purified plasmid were used to linearized with the restriction enzyme that do not cut the DNA insert but cut the vector by using *Apa*I (Biolab) and *Spe*I (Biolab), the reaction were carried out using 1x buffer enzyme of *ApaI*, 5 µg of plasmid DNA, 5 units of *ApaI* restriction enzyme and DW in total volume 100 µl for *ApaI* tube and 1x buffer enzyme of *SpeI*, 5 µg of plasmid DNA, 5 units of *SpeI* restriction enzyme and DW in total volume 100 µl for *SpeI* tube, then incubated each tube at 37 °C for 2-3 h and DNA precipitated with 3M sodium acetate pH 5.2 and absolute ethanol by inverting and incubated at -20 °C for 2 h or -80 °C for overnight then centrifuged at 13,000 rpm at 4 °C for 15 min and discarded supernatant. After that, DNA were washed with 70% ethanol, centrifuged at 13,000 rpm at 4 °C for 5 min. The pellet DNA were dried at room temperature for 5-10 min and resuspended with DEPC water by vortexing. The quality of the isolated RNA was then assessed using nano drop spectrophotometers (Thermo Scientific™).

The linearized plasmid was used as template to synthesize antisense and sense RNA probe by *in vitro* transcription using digoxigenin (DIG)-labeled uridine triphosphate (UTP) with T7 or SP6 RNA polymerase (Roche Diagnostics, Germany) according to the manufacturer's instructions. Briefly, 1 µg linear plasmid were mixed with 0.5 µl of DIG RNA labeling (10x conc.), 1.4 µl of T7 buffer (10x conc.), 0.5 µl of T7 or Sp6 RNA polymerase (20 U) , 0.5 µl of RNase Inhibitor (20 U) and DEPC water in total volume 14 µl, then incubated at 37°C for 2 h, treated with 2 µl of DNase (20 U) (Roche Diagnostics, Germany) at 37°C for 15 min and precipitated with ethanol by using 100 µl of 100% ethanol, 3 µl of 3M sodium acetate and 1 µl of 10 mg/ml Yeast tRNA. Then, RNA probe synthesis was checked by dot blot on paper and detected with NBT/BCIP. Antisense and sense probe were aliquoted to made concentration 1 µg/µl per tube and stored at -80°C until use.

2.3.5 *In situ* hybridization analysis

In situ hybridization (ISH) was performed on testes section of banana shrimp and black tiger shrimp. The fragment of a 741 and 846 base pair (bp) cDNA vasa for banana shrimp and black tiger shrimp, respectively.

For the tissue *in situ* hybridization, testes of banana shrimp and black tiger shrimp were fixed in Bouin's solution fixative at 4 °C for 7 h. After fixation, testes were dehydrated as follows: 70% ethanol for 1 h, 85% ethanol for 1 h, twice in 95% ethanol for 1 h, twice in 100% ethanol for 1 h, twice in xylene for 1 h, and twice in paraplast for 1.3 h. Then testes were embedded into paraffin wax, paraffin-embedded tissue samples were serial sectioned at 5 µm with a microtome, mounted on glass slides coated with 3-Aminopropyltriethoxysilane, deparaffinized and dehydrated in xylene-ethanol gradient. The paraffin sections were stained with hematoxylin-eosin (H&E) or ISH by hybridized with DIG-labeled RNA probes. For ISH, the paraffin sections were permeabilized with proteinase K for 30 min at 37 °C, acetylated, and hybridized at 65 °C for 18 h with a hybridization solution containing 1 µg/ml antisense or sense DIG-

labeled RNA probe, 50% (v/v) of formamide, 10% (v/v) of 20x saline sodium citrate (SSC) (pH 4.5), 50 µg/ml transfer RNA (tRNA), 50 µg/ml heparin, 2% (v/v) of sodium dodecyl sulfate (SDS) (10%), and 40% (v/v) of dextran sulfate (25%). After hybridization, the sections were washed as follows: twice in 2x SSC/50% formamide for 30 min at 65 °C, three times in 1x SSC/50% formamide for 20 min at 65 °C, and once in 0.5x SSC/25% formamide for 10 min at 65 °C. To reduce background, the sections were treated with 10 µg/ml RNase A for 10 min at 37 °C to digest nonspecific binding probes. After RNase digestion, the sections were washed twice in NTE buffer (5 M NaCl, 2 M Tris-HCl pH 8.0, 0.5 M EDTA pH 8.0) at 37 °C for 5 min, three times in 0.5x SSC at 65 °C for 20 min, cool samples at RT for 30 min and then washed three times in 1x Tris-buffered saline containing 0.1% Tween 20 (TBST) for 5 min at RT. Non specific binding was blocked with 2% blocking reagent (Roche Diagnostics, Germany) /TBST for 15 min in moist chambers at RT. The sections were incubated with anti-DIG-AP Fab fragments (diluted 1:500 in the blocking reagent) for 2 h at RT, then washed three times in PBST followed by once in NTMT solution (5 M NaCl, 2 M Tris–HCl pH 9.5, 1 M MgCl2, 0.1% Tween 20) for 5 min at RT. Hybridization signals were then detected by incubation in NTMT solution containing nitroblue tetrazolium (NBT; Roche Diagnostics, Germany) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP; Roche) and placed in dark area at RT. After detection the signals, the section slides were mounted using Entellan® New (Merck, Darmstadt, Germany) and observed the results under fluorescence microscope.

2.3.6 Cryopreservation experiment

2.3.6.1 Optimal incubation time for slow freezing method

To optimize incubation time of testes in cryomedium containing cryoprotectant for slow freezing method. Dissected testes (0.018-0.02 g) of giant freshwater prawn were transferred into 1.8 ml cryovials (NEST®) containing 500 µl cryomedium contained cryoprotectant (DMSO, GLY and Magnesium chloride ($MgCl_2$)), 100 mM Trehalose,

15% Bovine serum albumin (BSA) (Sigma, USA) and 2x extender contained 10x extender (19 mM hydroxyethyl piperazineethanesulfonic acid (HEPES), 130 mM Sodium chloride (NaCl), 2 mM Potassium chloride (KCL), 8 mM Potassium hydrogen phosphate (KH_2PO_4), 1 mM Sodium hydrogen phosphate (Na_2HPO_4) and 1 mM Sodium pyruvate), 0.9 mM Calcium chloride dehydrate ($CaCl_2 \cdot 2H_2O$) and 0.5 mM $MgCl_2 \cdot 6H_2O$. Samples were incubated for 15, 30 and 60 min on ice, and cooled at -1 °C/min for 90 min using a freezing container (Bicell; Nihon Freezer Co., Ltd., Japan) located in a -80 °C. After cooling, the samples were plunged into liquid nitrogen (-196 °C) and were stored for a week before analysis. Freshly testes were used as a control, five replicates were prepared (N=5).

2.3.6.2 Effect of cryoprotectant type and concentration on spermatogonia cells viability

To evaluate the toxicity of type and concentration of cryoprotectants for cryopreservation protocol; 5, 10 and 15% of DMSO, GLY and $MgCl_2$ were used as cryoprotectant. For a slow freezing method, dissected testes (0.018-0.02 g) were transferred into 1.8 ml cryovials containing 500 µl cryomedium contained cryoprotectant, 100 mM trehalose, 15% BSA and 2x extender contained 10x extender (19 mM HEPES, 130 mM NaCl, 2 mM KCL, 8 mM KH_2PO_4, 1 mM Na_2HPO_4 and 1 mM Sodium pyruvate), 0.9 mM $CaCl_2 \cdot 2H_2O$ and 0.5 mM $MgCl_2 \cdot 6H_2O$. Samples were incubated for 60 min on ice and cooled at -1 °C/min for 90 min using a freezing container located in a -80 °C. After cooling, the samples were plunged into liquid nitrogen (-196 °C) and were stored for a week. For a vitrification method, the testes were placed on copper mesh (Clever, Aichi, Japan) and then transferred into 24 well plate containing 1 ml vitrification solution contained cryoprotectant, 200 mM trehalose, 15% of Ethylene glycol, 20% of Fetal bovine serum (FBS)

(Gibco Invitrogen Co.) and 3.3x extender contained 10x extender (26.8 mM HEPES, 182.1 mM NaCl, 3.5 mM KCL, 11.2mM KH_2PO_4, 1.5 mM Na_2HPO_4 and 1.75 mM Sodium pyruvate), 1.3mM $CaCl_2 \cdot 2H_2O$ and 0.7 $MgCl_2 \cdot 6H_2O$. After incubation for 30 min on ice, samples were removed from vitrification solution, transferred into 1.8 ml transparent cryovials and then the samples were plunged immediately into liquid nitrogen (-196 ^oC) and were stored for a week (Seki et al. 2017). In both methods, freshly testes were used as a control. Five replicates were prepared (N=5).

2.3.6.3 Effect of thawing temperature on spermatogonia cells viability

To optimize thawing temperature for slow freezing and vitrification methods, the testes were cryopreserved with 10% DMSO and 10% GLY for banana shrimp and black tiger shrimp, respectively. For slow freezing method, the frozen testes in cryovial were thawed at 10 ^oC and 27 ^oC by shaking cryovial in a water bath and for vitrification method, the piece of frozen testes placed on copper mesh were taken out from cryovial and thawed in Leibovitz's L-15 medium at 10 ^oC and 27 ^oC and removed from copper mesh. Then, thawed testes of both methods were rehydrated in three times by shaking in Leibovitz's L-15 medium (pH 7.8) containing 27.75 mM D-Glucose, 10% FBS and 1% antibiotic–antimycotic and dissociated. To assess spermatogonia cell viability, the testes cells were stained with the trypan blue (Gibco, USA) and counted using a hematocytometer under a light microscopy, five replicates were prepared (N=5).

2.3.6.4 Long-term cryopreservation

The ultimate purpose of this study was to optimize the conditions for long-term cryopreservation of testes of shrimp, 10% DMSO were used

for long-term cryopreservation of giant freshwater prawn and banana shrimp and 10% GLY were used for long-term cryopreservation of black tiger shrimp.

Control group was freshly dissected testes (0.018-0.02 g) collected from shrimp. In the experiment group of slow freezing method, samples were transferred into cryovial containing 500 µl cryomedium contained cryoprotectant, 100mM trehalose, 15% BSA and extender. Samples were equilibrated for 60 min on ice and cooled at -1 °C/min for 90 min using a freezing container located in a -80 °C. And in the experiment group of vitrification method, samples were placed on copper mesh and then transferred into 24 well plate containing 1 ml vitrification solution contained cryoprotectant, 200mM trehalose, 15% Ethylene glycol, 20% FBS and extender. Samples were equilibrated for 30 min in vitrification solution on ice, then were transferred into transparent cryovials. The samples of slow freezing and vitrification methods were plunged into liquid nitrogen and were stored at 1, 2, 3 and 6 months, then testes were thawed at 10 °C. Five replicates were prepared (N=5).

2.3.6.5 Dissociation procedure

To assess spermatogonia cell viability, after the frozen testes were thawed and rehydrated in three changed Leibovitz's L-15 medium (pH 7.8) containing 27.75 mM D-Glucose, 10% FBS and 1% antibiotic–antimycotic. The frozen testes were dissociated in Leibovitz's L-15 medium (pH 7.8) containing 1.665 mg/ml of dispase II (33.3 mg/ml) (Sigma, USA), 2 mg/ml of collagenase H (40mg/ml) (Roche, Germany) and 0.45 u/ul of DNase I (15 u/µl) (Sigma, USA) in total volume 500 µl by cutting for 5 min and pipetting every 30 min for 1 h at 25 °C. The cell suspension was inactivated of enzyme by adding Leibovitz's L-15 medium (pH 7.8) containing 27.75mM D-Glucose, 10% FBS and 1% antibiotic–antimycotic and filtered through a 42 µm-pore nylon screen

and centrifuged at 300 g for 10 min. The harvested cells were resuspended with Leibovitz's L-15 medium (pH 7.8) containing 27.75mM D-Glucose, 10% FBS and 1% antibiotic–antimycotic, stained with the trypan blue; ratio 1:1 and counted using a hematocytometer under a light microscopy. The dead cells were stained blue and the live cells were unstained. The calculation followed the formula described by Octavera and Yoshizaki (2020): cell viability (%) = number of trypan blue-negative cells / total number of cells (trypan blue-positive and blue-negative cells) × 100. The rate of cell recovery was calculated as the percentage of live cells isolated from the cryopreserved tissue compared with the number of live cells isolated from the fresh tissue, while correcting for the tissue size using the following formula: recovery rate (%) = (total live cryopreserved cells / total live fresh cells) × (weight of fresh tissue / weight of cryopreserved tissue) × 100. All data were obtained from five independent experiments.

2.3.7 Flow cytometry

Dissected testes (0.018-0.02 g) were preserved with 10% DMSO for giant freshwater prawn and banana shrimp and 10% GLY for black tiger shrimp at 1-3 months. Then, cryopreserved testes were thawed at 10 °C, dissociated and spermatogonia cells suspension were prepared in Guava Nexin Reagent (Merck), following the manufacturer's protocols of Guava Nexin Assay by using flow cytometer (Flow Cytometer Guava EasyCyte 8HT). Spermatogonia cells suspension were incubated with Guava Nexin Reagent by pipetting 100 µl of spermatogonia cell suspension into 96 well, adding 100 µl of Guava Nexin Reagent to 96 well and then, incubate for 20 min at room temperature in the dark. Twenty-five thousand events were recorded, and cell populations were determined based on cell size. Percentage of apoptotic cells were recorded, and

percentage of viable cells, early apoptosis, late apoptosis, and debris cells were analyzed with Guava System.

2.3.8 Statistical analysis

The results were expressed as mean ± standard deviation (SD). The data were tested for normal distribution and equal variances by using SPSS program. Data were analyzed using repeated measures one-way analysis of variance (ANOVA) followed by Tukey's post hoc test. All analyses were performed at a significance level $P < 0.05$.

CHAPTER 3

RESULTS

3.1 Immunohistochemical analysis

The testes of giant freshwater prawn were identified the germ cells developmental stages by immunohistochemical analysis which performed on testes section of giant freshwater prawn, some deparaffinized testes slides were stained with hematoxylin and eosin (H&E), which is tissue stains used in histology (Figure 10 A). The protein vasa, which is a germ cell marker, was immunohistochemically stained on adjacent deparaffinized testes slides (Figure 10 B). After deparaffinized testes slides were incubated with polyclonal rabbit anti-vasa antibody (1^{st} antibody) and Alexa Fluor 488-conjugated goat anti rabbit IgG, which is used for the indirect detection of a target to which a specific primary antibody is first bound.

Testes slides were immunohistochemically stained with anti-Vasa antibody, the signals were specifically detected in only spermatogonia cell (SG) but not detected in spermatocyte (SC) and sperm (S) as morphology of germ cell stages were identified according to the classification reported in the giant freshwater prawn (Poljaroen et al. 2010).

Spermatogonia

Spermatogonia are found on the outside edges of seminiferous tubules. A spermatogonium is an oval-shaped cell with one or two large nucleoli and a large centrally placed nucleus containing predominantly euchromatin. Ribosomes and mitochondria abound throughout the cytoplasm. Nurse cells can also be found near the spermatogonia in the tubule's periphery.

Spermatocyte

Primary spermatocytes are spherical cells with moderately basophilic nuclei and patchy heterochromatin dispersed throughout, measuring 10-14 µm in diameter. Primary spermatocytes can be divided into six phases (Leptotene spermatocyte (LSc), Zygotene spermatocyte (ZSc), Pachytene spermatocyte (PSc), Diplotene spermatocyte (DSc), Diakinesis spermatocyte (DiSc), Metaphase spermatocyte (MSc)) based on the arrangement of chromatin fibers. The first meiotic division of a primary spermatocyte generates two secondary spermatocytes with highly condensed cord-like components that are collected together in its chromatin.

Sperm

A testicular sperm is composed of a dense anterior part with a spike-like projection and its base plate, like an everted umbrella, and a globular and transparent posterior part. It spans 8-9 µm across the globular part's midsection, which includes the nucleus surrounded by a thin cytoplasmic border.

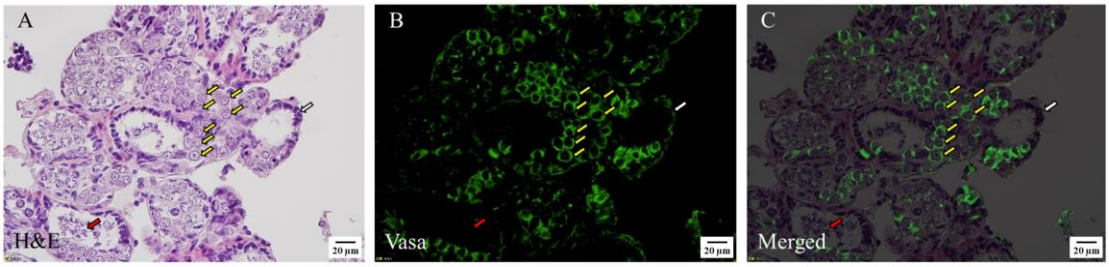


Figure 10. Immunohistochemistry with anti-Vasa antibody in the testes of giant freshwater prawn. Paraffin sections of testes were stained with hematoxylin and eosin (A), immunohistochemically stained with anti-Vasa antibody (B), and merged (C). Antibody signals were detected on spermatogonia cells (yellow arrow) but not detected on spermatocyte (white arrow) and sperm (red arrow). Bars represent 20 µm.

3.2 Tissue distribution analysis by RT-PCR

For analysis the expression of vasa in different tissue of adult banana shrimp and black tiger shrimp. RT-PCR was used to determine the tissue distribution patterns of vasa RNA in adult tissue (testes , muscle , gill , hepatopancreas , heart , intestine , brain , thoracic ganglion and lymphoid) and RNA was used for cDNA synthesis using *vasa*-specific primer, which generate amplicons of 741 bp and 846 bp for banana shrimp (Figure 11 A) and black tiger shrimp (Figure 11 B), respectively and *β*-actin was amplified as an internal control and no cDNA template as a negative control. The abbreviation MW stands for molecular weight marker (100-bp DNA ladder).

The results of banana shrimp and black tiger shrimp showed specially expressed predicted molecular weight of 741 bp and 846 bp in the testes, respectively and was not expressed in other tissues including muscle, gill, hepatopancreas, heart, intestine, brain, thoracic ganglion and lymphoid.

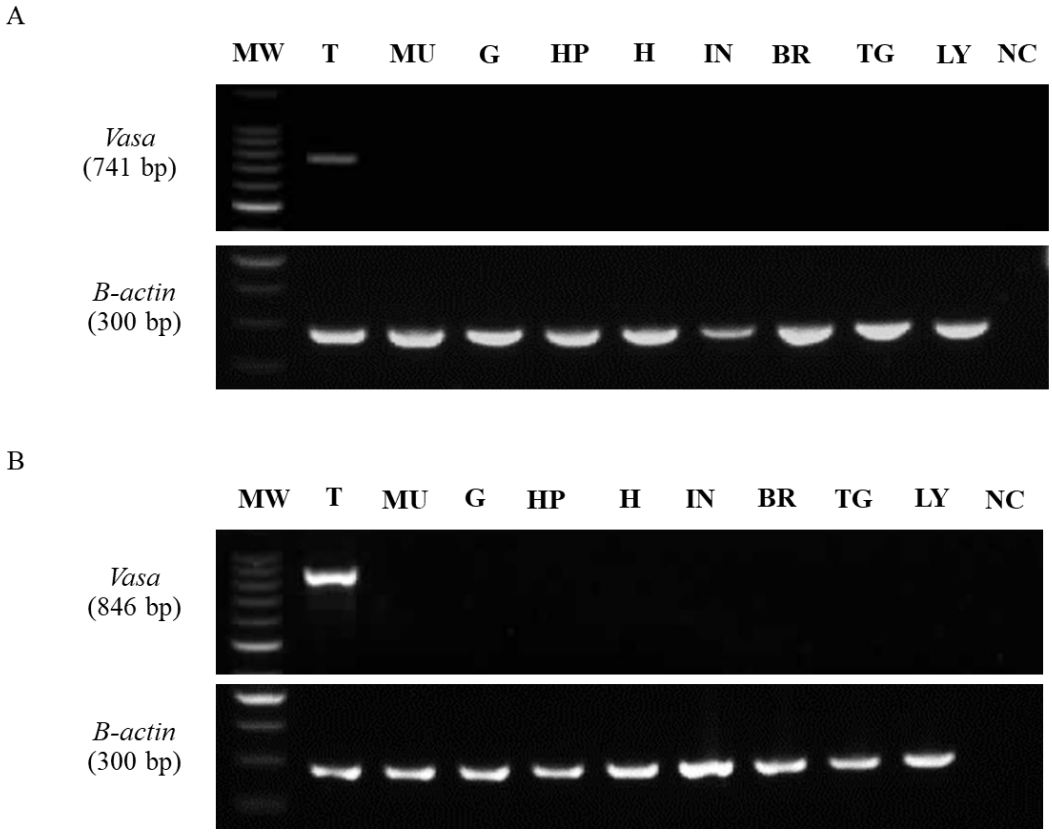


Figure 11. RT-PCR analysis of *vasa* mRNA expression in banana shrimp (A) and black tiger shrimp (B). cDNAs were synthesized using total RNA isolated from various tissues (testes (T), muscle (MU), gill (G), hepatopancreas (HP), heart (H), intestine (IN), brain (BR), thoracic ganglion (TG) and lymphoid (LY)) using *vasa*-specific primer, which generate amplicons of 741 bp and 846 bp for banana shrimp and black tiger shrimp, respectively and *β*-actin was amplified as an internal control. There was no cDNA template in Lane NC, which served as a negative control. The abbreviation MW stands for molecular weight marker (100-bp DNA ladder).

3.3 *In situ* hybridization analysis

In situ hybridization (ISH) was used to identify stage of spermatogonia cells expression in adult testes of banana shrimp (Figure 12 A-C) and black tiger shrimp (Figure 12 D-F) by using *vasa* RNA probes, which used for identification stage of spermatogonia cell expressed in adult testes of banana shrimp and black tiger shrimp. ISH was performed on testes section, some deparaffinized testes slides were stained with hematoxylin and eosin, which is tissue stains used in histology and were hybridized with antisense and sense DIG-labeled *vasa* RNA probes.

The results of hematoxylin and eosin stains showed mainly stages of spermatogonia and spermatid in testes of both shrimp as follow described about the reproductive morphology of penaeoidean shrimps (Fransozo et al. 2016). The signals of *vasa* RNA were specifically detected in only spermatogonia cell of banana shrimp (Figure 12 A-A') and black tiger shrimp (D-D'), no signals of vasa RNA were detected in spermatid. Conversely, no hybridization signals were observed in any of the cells of banana shrimp (Figure 12 B-B') and black tiger shrimp (E-E') when the sense probes were performed on testes section.

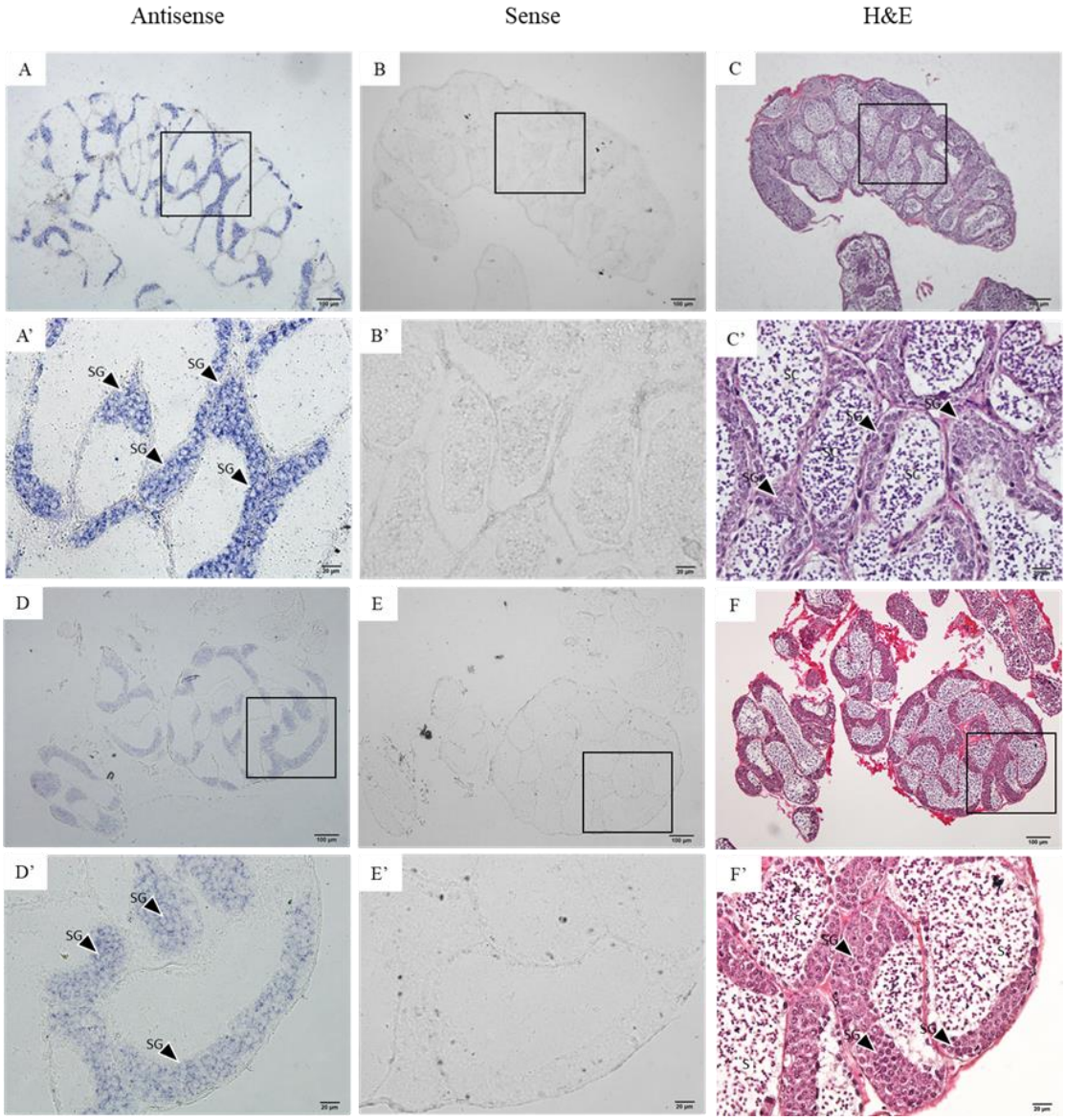


Figure 12. Histological characterization and *vasa* mRNA expression in testes of banana shrimp (A-C) and black tiger shrimp (D-F) by *in situ* hybridization using vasa antisense and sense probes. Sequential sections hybridized with an antisense vasa probe (A and D), sense vasa probe (B and E) and stained with H&E (C and H). No signal is detected with sense probe. The *vasa* mRNA signal is specifically expressed in spermatogonia cell (SG). Picture (A', B' and C') is high magnification of the black region of picture (A, B and C,) and picture (D', E' and F') is high magnification of the black region of picture (D, E and F), respectively. Scale bars represents 50 µm (A-F) and 20 µm (A'-F'). SG; spermatogonia (arrowheads) and S; spermatid.

3.4 Cryopreservation experiment of testes

3.4.1 Effect of equilibration times in cryoprotectants on viability and recovery of spermatogonia cell

To determine the suitability equilibration times in cryoprotectants of shrimp, testes of giant freshwater prawn was incubated in cryomedium containing 10% DMSO, 10% GLY and 10% $MgCl_2$ and extender for 15, 30 and 60 min, and cryopreserved by using slow freezing method. The viability of spermatogonia cells incubated with 10% DMSO for 15, 30 and 60 min was 97.45 ± 2.68, 96.81 ± 3.45 and 94.32 ± 4.23%, 10% GLY was 92.78 ± 3.75, 90.91 ± 2.81 and 88.42 ± 3.04% and 10% $MgCl_2$ was 93.71 ± 2.92, 96.59 ± 3.23 and 89.53 ± 3.65%, respectively (Figure 13 A). The recovery of spermatogonia cells incubated with 10% DMSO for 15, 30 and 60 min was 96.48 ± 3.61, 93.74 ± 3.05 and 92.99 ± 3.32%, 10% GLY was 87.05 ± 3.22, 89.51 ± 4.78 and 84.12 ± 4.49% and 10% $MgCl_2$ was 89.80 ± 4.38, 93.28 ± 2.97 and 84.67 ± 5.30%, respectively (Figure 13 B). In each of equilibration times in cryoprotectants on viability and recovery of spermatogonia cell was not significantly different.

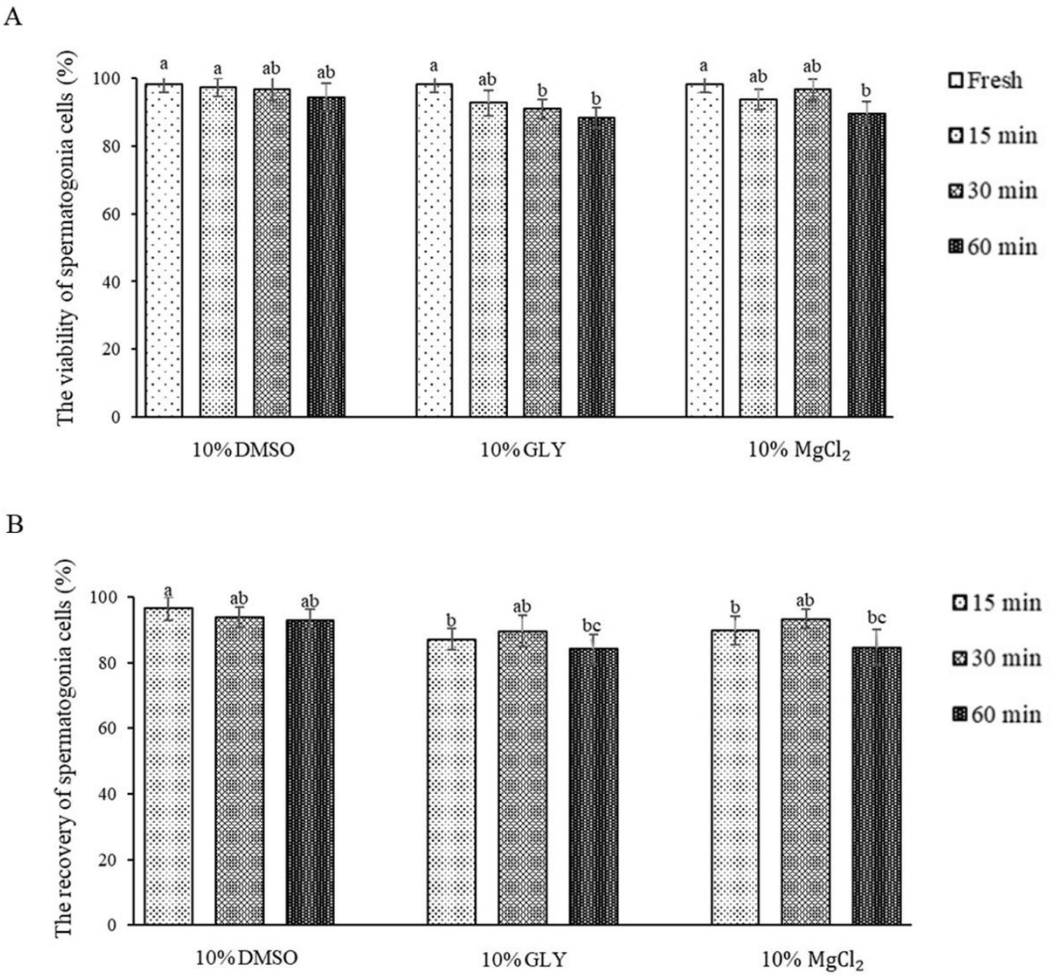


Figure 13. Optimization of equilibrate conditions by using slow freezing method for spermatogonia cell of giant freshwater prawn. (A) The viability of spermatogonia cell (%) and (B) The recovery of spermatogonia cell (%) equilibrated for 15, 30 and 60 min on ice with cryomedium containing cryoprotectants at 10% concentrations. Each bar represents the mean ± SD (N=5).

3.4.2 Effect of cryoprotectant type and concentration on viability and recovery of spermatogonia cell

3.4.2.1 Effect of cryoprotectant type on viability and recovery of spermatogonia cell

To preserve the cells and retain high viability and recovery of spermatogonia cells, it is essential to prevent intracellular ice formation during cooling and warming by dehydration and permeation of cryoprotectants including DMSO, GLY and $MgCl_2$. For slow freezing method, the viability and recovery of spermatogonia cells of giant freshwater prawn were preserved using 10% of DMSO, GLY and $MgCl_2$was 87.84 ± 2.74 and 75.58 ± 4.30%, 85.39 ± 2.68 and 67.46 ± 4.20%, and 82.78 ± 4.56 and 62.41 ± 8.68%, respectively, in each cryoprotectant was not significantly different. The viability and recovery of spermatogonia cells of banana shrimp were preserved using 10% of DMSO was 90.68 ± 4.77 and 58.85 ± 2.69% and GLY was 82.37 ± 8.45 and 42.26 ± 5.55% and black tiger shrimp were preserved using 10% of DMSO was 87.27 ± 2.55 and 82.92 ± 8.12% and GLY was 86.17 ± 2.08 and 89.58 ± 8.96%, respectively, was significantly higher than that preserved using 10% of $MgCl_2$ (Figure 14 A, B). For vitrification method, the viability of spermatogonia cells preserved using 10% of DMSO and GLY of giant freshwater prawn was 90.87 ± 1.39 and 84.45 ± 2.49%, banana shrimp was 80.70 ± 3.22 and 76.40 ± 3.05%, and black tiger shrimp was 85.66 ± 6.99 and 79.37 ± 4.31%, respectively and recovery of spermatogonia cells preserved using 10% of DMSO and GLY of giant freshwater prawn was 71.79 ± 3.29 and 71.02 ± 6.04%, banana shrimp was 53.69 ± 7.95 and 39.87 ± 5.56%, and black tiger shrimp was 85.00 ± 5.97 and 86.67 ± 8.15%, respectively, was significantly higher than that preserved using 10% of $MgCl_2$ (Figure 14 C, D). However, when testes in banana shrimp were preserved by using slow freezing and vitrification methods with 10% of DMSO (58.85 ± 2.69, 53.69 ± 7.95%), the recovery of spermatogonia cells was

significantly higher than that of preserved in 10% of GLY (42.26 ± 5.55, 39.87 ± 5.56%), respectively. But the recovery of spermatogonia cells of giant freshwater prawn were preserved with 10% of DMSO (75.58 ± 4.30 and 71.79 ± 3.29%) and 10% of GLY (67.46 ± 4.20 and 71.02 ± 6.04%) and black tiger shrimp were preserved with 10% of DMSO (82.92 ± 8.12 and 85.00 ± 5.97%) and 10% of GLY (89.58 ± 8.96 and 86.67 ± 8.15%), was not significantly different for slow freezing and vitrification methods, respectively (Figure 14 B, D).

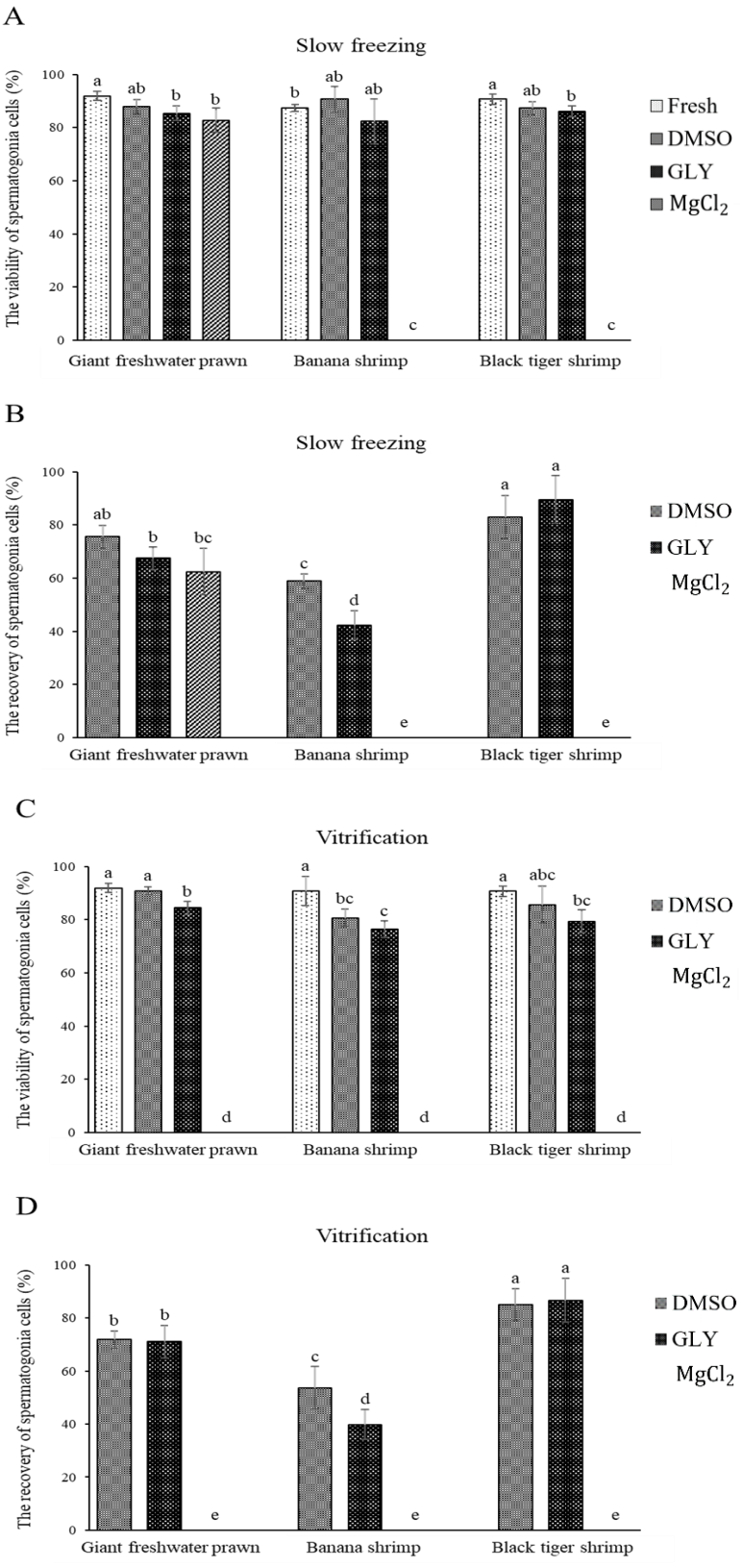

A
Slow freezing
The viability of spermatogonia cells (%)
Giant freshwater prawn
Banana shrimp
Black tiger shrimp
Fresh
DMSO
GLY
MgCl2
B
Slow freezing
The recovery of spermatogonia cells (%)
DMSO
GLY
MgCl2
C
Vitrification
The viability of spermatogonia cells (%)
DMSO
GLY
MgCl2
D
Vitrification
The recovery of spermatogonia cells (%)
DMSO
GLY
MgCl2

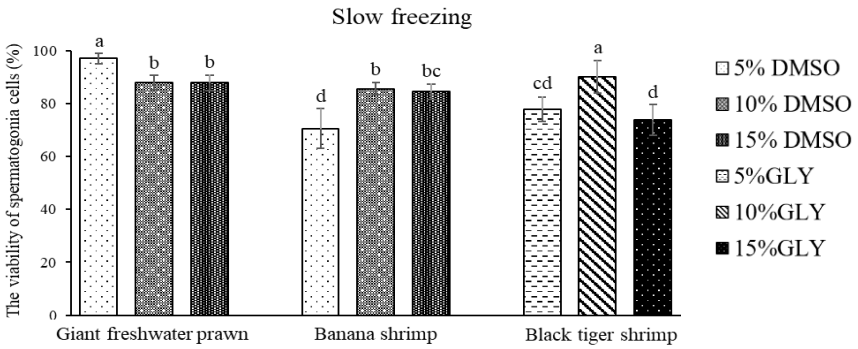
Figure 14. Optimization of cryoprotectant type condition for giant freshwater prawn, banana shrimp and black tiger shrimp spermatogonia by using slow freezing and vitrification method. (A and C) The viability of spermatogonia cell (%) and (B and D) the recovery of spermatogonia cell (%) after freezing with cryomedium containing 10% of dimethyl sulfoxide (DMSO), glycerol (GLY) and Magnesium chloride ($MgCl_2$). The control group, freshly testes were dissociated in L-15 medium without cryoprotectants. Each bar represents the mean ± SD (N=5).

3.4.2.2 Effect of cryoprotectant concentration on viability and recovery of spermatogonia cell

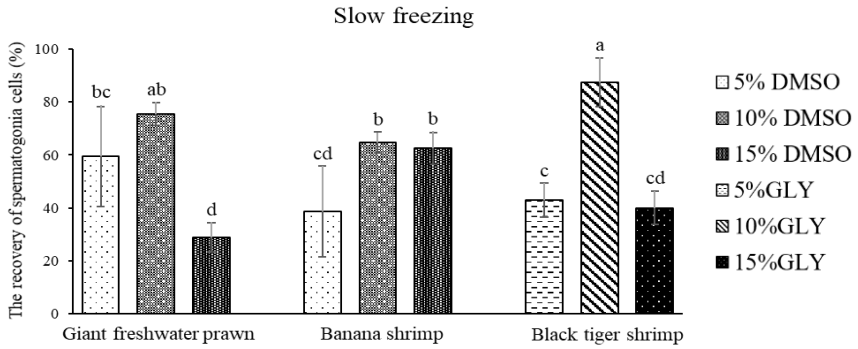
Based on effect of cryoprotectant type result, concentrations at 5%, 10% and 15% of DMSO for giant freshwater prawn and banana shrimp and GLY for black tiger shrimp were observed by using slow freezing and vitrification methods. For slow freezing method, the viability of spermatogonia cells of giant freshwater prawn were preserved using 5% of DMSO (96.98 ± 2.00%) was significantly higher than using 10% of DMSO (87.93 ± 2.74%) and 15% of DMSO (87.77 ± 2.85%), banana shrimp were preserved using 10% of DMSO (85.59 ± 2.39%) and 15% of DMSO (84.44 ± 2.94%) was significantly higher than using 5% of DMSO (70.51 ± 7.43%) and black tiger shrimp were preserved using 10% of GLY (90.01 ± 6.12%) was significantly higher than using 5% of GLY (77.77 ± 4.46%) and 15% of GLY (73.68 ± 5.82%) (Figure 15 A). For vitrification method, the viability of spermatogonia cells of giant freshwater prawn were preserved using 5% of DMSO (94.23 ± 3.26%) and 10% of DMSO (88.59 ± 4.31%) was significantly higher than using 15% of DMSO (82.98 ± 2.89%) but banana shrimp were preserved using 5%, 10% and 15% of DMSO (81.45 ± 4.26, 83.28 ± 3.81 and 87.19 ± 8.44%) and black tiger shrimp were preserved using 5%, 10% and 15% of GLY (85.38 ± 6.36, 86.50 ±

3.66 and 85.21 ± 5.29%), respectively, was not significantly different (Figure 15 C). However, the recovery of spermatogonia cells were preserved by using slow freezing and vitrification methods with 10% of DMSO for giant freshwater prawn (75.58 ± 4.30 and 83.38 ± 3.29%), and banana shrimp (64.65 ± 3.94 and 61.15 ± 8.83%) and 10% of GLY for black tiger shrimp (87.50 ± 9.32 and 90.83 ± 8.67%), respectively, was highest (Figure 15 B, D).

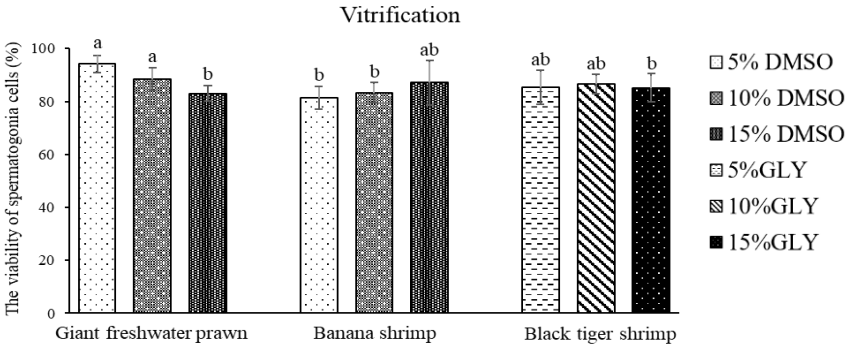
A


Slow freezing
The viability of spermatogonia cells (%)
100
80
60
40
20
0
a
b
b
d
b
bc
cd
a
d
Giant freshwater prawn
Banana shrimp
Black tiger shrimp
5% DMSO
10% DMSO
15% DMSO
5%GLY
10%GLY
15%GLY

B


Slow freezing
The recovery of spermatogonia cells (%)
100
80
60
40
20
0
bc
ab
d
cd
b
b
c
a
cd
Giant freshwater prawn
Banana shrimp
Black tiger shrimp
5% DMSO
10% DMSO
15% DMSO
5%GLY
10%GLY
15%GLY

C


Vitrification
The viability of spermatogonia cells (%)
100
80
60
40
20
0
a
a
b
b
b
ab
ab
ab
b
Giant freshwater prawn
Banana shrimp
Black tiger shrimp
5% DMSO
10% DMSO
15% DMSO
5%GLY
10%GLY
15%GLY

D

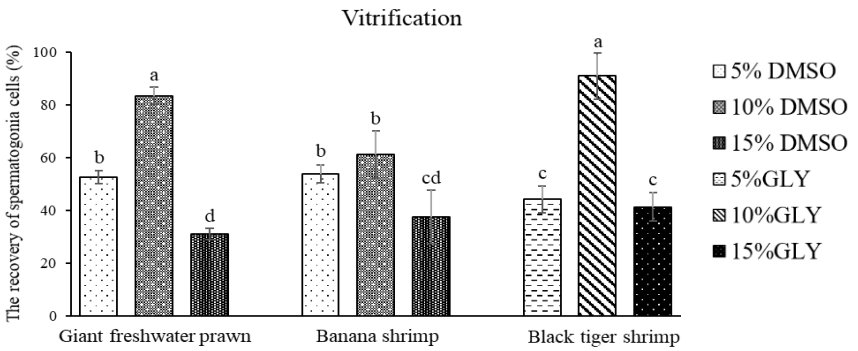

Vitrification
The recovery of spermatogonia cells (%)
100
80
60
40
20
0
b
a
d
b
b
cd
c
a
c
Giant freshwater prawn
Banana shrimp
Black tiger shrimp
5% DMSO
10% DMSO
15% DMSO
5%GLY
10%GLY
15%GLY

Figure 15. Optimization of cryoprotectant concentrations for giant freshwater prawn, banana shrimp and black tiger shrimp spermatogonia by using slow freezing and vitrification method. (A and C) The viability of spermatogonia cell (%) and (B and D) the recovery of spermatogonia cell (%) after freezing with different concentrations (5%, 10% and 15%) of cryomedium containing DMSO for giant freshwater prawn and banana shrimp and glycerol (GLY) for black tiger shrimp. Each bar represents the mean ± SD (N=5).

3.4.3 Effect of thawing temperature on viability and recovery of spermatogonia cell

In thawing experiments, the frozen spermatogonia cells were preserved in liquid nitrogen (-196 °C) by using slow freezing and vitrification methods. For slow freezing method, the viability of spermatogonia cells of giant freshwater prawn and banana shrimp were thawed at 10 °C (92.26 ± 2.82 and 84.78 ± 3.26%) was significantly higher than thawed at 27 °C (78.92 ± 3.64 and 76.29 ± 4.12%), respectively, and the viability of spermatogonia cells of black tiger shrimp was not significantly different between 10 °C (83.72 ± 3.53%) and 27 °C (69.06 ± 14.76%) (Figure 16 A). For vitrification methods, the viability of spermatogonia cells of giant freshwater prawn and black tiger shrimp was not significantly different between 10 °C (91.38 ± 3.89 and 82.80 ± 3.62%) and 27 °C (88.34 ± 2.28 and 83.33 ± 5.80%), respectively and the viability of spermatogonia cells of banana shrimp were thawed at 10 °C (86.96 ± 3.42%) was significantly higher than thawed at 27 °C (70.18 ± 3.04%) (Figure 16 C). However, the recovery of spermatogonia cells in giant freshwater prawn, banana shrimp and black tiger shrimp thawed at 10 °C (91.23 ± 1.56, 79.20 ± 4.69, 89.58 ± 5.71% and 89.63 ± 3.24, 83.04 ± 2.93, 88.33 ± 6.69%) was significantly higher than at 27 °C (50.12 ± 2.78, 34.66 ± 3.71, 20.42 ± 5.78% and 57.86 ± 3.11, 40.51 ± 6.04, 21.25 ± 5.78%) for slow freezing and

vitrification methods, respectively (Figure 16 B, D). Thus, thawing temperature of 10 °C was suitable for thawing the frozen testes.

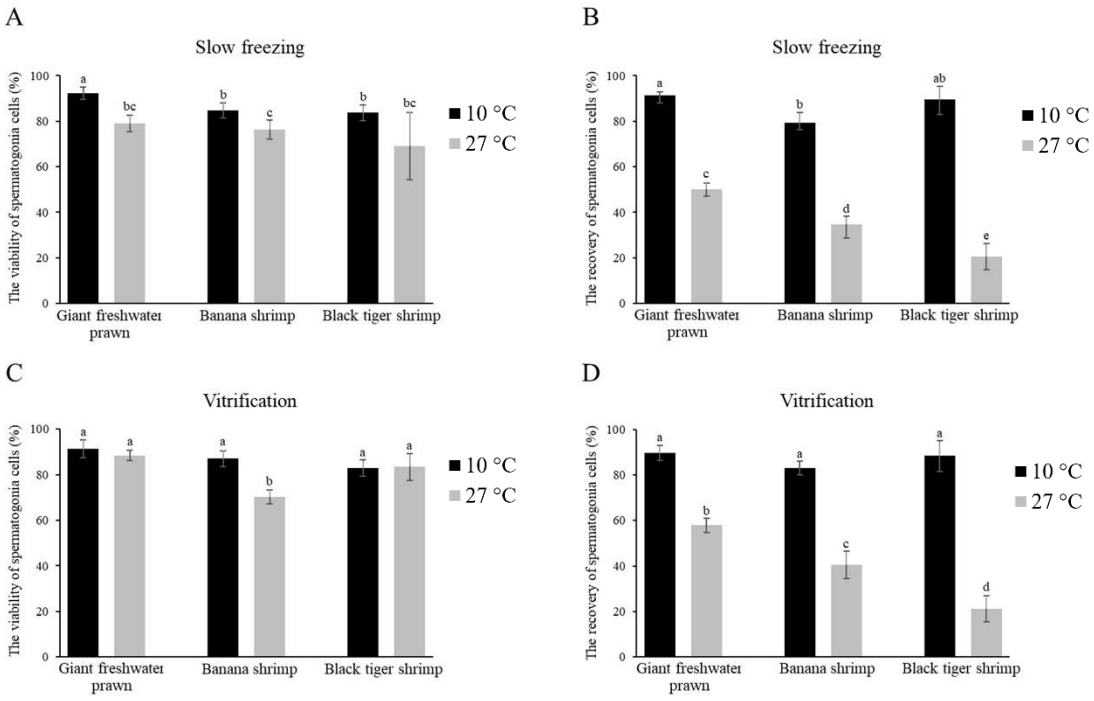


Figure 16. Optimization of thawing temperature for giant freshwater prawn, banana shrimp and black tiger shrimp of frozen-thawed spermatogonia by using slow freezing and vitrification method. (A and C) The viability of spermatogonia cell (%) and (B and D) the recovery of spermatogonia cell (%) after thawed at 10 °C and 27 °C. Each bar represents the mean ± SD (N=5).

3.4.4 Long-term cryopreservation

For long-term cryopreservation of spermatogonia cells, the dissect testes were preserved at 1, 2, 3 and 6 months by using slow freezing and vitrification methods. In giant freshwater prawn, the viability of spermatogonia cells at 1 month (89.37 ± 2.12 and 94.29 ± 1.85%) was not significantly different with 6 months (84.13 ± 2.49 and 92.03 ± 2.63%) for slow freezing and vitrification method, respectively (Figure 17 A) and the recovery of spermatogonia cells at 1 month (72.94 ± 3.18%) was significantly higher than at 6 months (50.93 ± 6.61%) for slow freezing but not significantly different between 1 month (94.80 ± 4.44) and 6 months (86.42 ± 5.88%) for vitrification method (Figure 17 B). In banana shrimp, the viability of spermatogonia cells at 1 month (85.50 ± 2.33 and 91.10 ± 1.69%) was not significantly different with 6 months (82.50 ± 2.28 and 88.51 ± 3.14%) for slow freezing and vitrification method, respectively (Figure 17 C). Whereases, the recovery of spermatogonia cells at 1 month (54.66 ± 4.29 and 89.31 ± 6.25%) was significantly higher than at 6 months (40.99 ± 7.69 and 71.02 ± 3.07%) for slow freezing and vitrification method, respectively (Figure 17 D). In black tiger shrimp, the viability of spermatogonia cells at 1 month (86.92 ± 2.53 and 89.07 ± 2.60%) was not significantly different with 6 months (84.12 ± 4.66 and 87.68 ± 2.01%) for slow freezing and vitrification method, respectively (Figure 17 E) and the recovery of spermatogonia cells at 1 month (91.21 ± 7.62 and 92.04 ± 4.60%) was higher than at 6 months (60.81 ± 4.93 and 77.91 ± 6.48%) for slow freezing and vitrification method, respectively (Figure 17 F). However, the recovery of spermatogonia cells preserved with vitrification method for long-term storage period at 6 months were significantly higher than of preserved with slow freezing method in all shrimps.

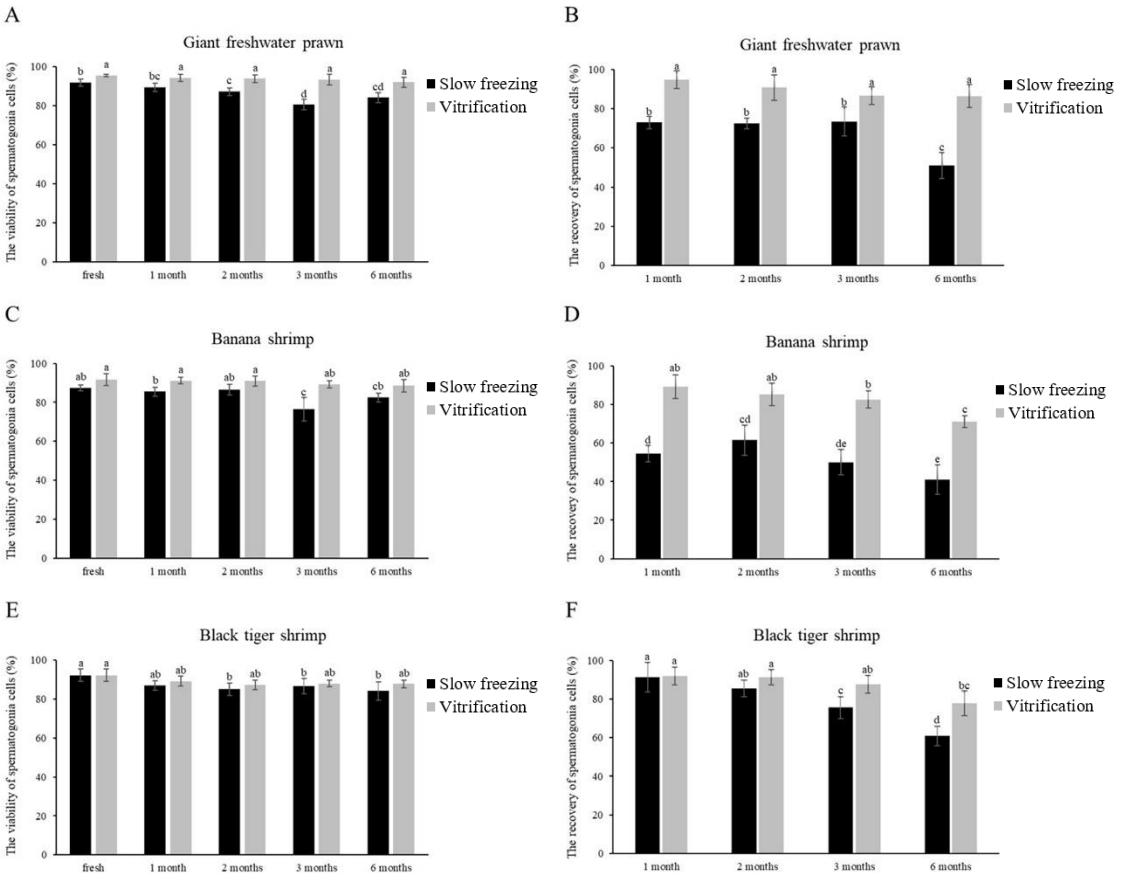


Figure 17. Comparison viability and recovery of spermatogonia cells between slow freezing and vitrification methods for giant freshwater prawn (A and B), banana shrimp (C and D) and black tiger shrimp (E and F), which preserved in liquid nitrogen for long-term storage period up to 6 months. The control group, freshly testes were dissociated in L-15 medium without cryopreservation. Each bar represents the mean ± SD (N=5).

3.5 Flow cytometry

To determine apoptosis cells from cryoprotectants toxicity after preserved in liquid nitrogen at 1-3 months with slow freezing and vitrification methods. Percentage of apoptosis cell identified by the Guava Nexin Reagent and analysed with the Guava Nexin Assay utilizes Annexin V-PE to detect PS on the external membrane of apoptotic cells. The cell impermeant dye, 7-AAD, is also used in the Guava Nexin Assay as an indicator of cell membrane structural integrity. In giant freshwater prawn, the viable cells (73.95 ± 1.33 and 74.80 ± 9.69%) were significantly higher than early apoptosis (0.19 ± 0.07 and 0.37 ± 0.06%), late apoptosis (0.23 ± 0.07 and 0.97 ± 0.49%) and debris cells (25.62 ± 1.41 and 23.9 ± 9.30%) for both slow freezing and vitrification methods, respectively (Figure 18 A). In banana shrimp, the viable cells (79.17 ± 3.30 and 82.53 ± 7.52%) was significantly higher than early apoptosis (1.23 ± 0.67 and 0.60 ± 0.26%), late apoptosis (0.87 ± 0.29 and 0.90 ± 0.44%) and debris cells (18.73 ± 3.70 and 16.00 ± 7.96%) for both slow freezing and vitrification methods, respectively (Figure 18 B). In black tiger shrimp, the viable cells (76.66 ± 3.46 and 86.50 ± 5.57%) was significantly higher than early apoptosis (2.70 ± 2.33 and 1.03 ± 0.71%), late apoptosis (1.73 ± 1.63 and 0.97 ± 0.67%) and debris cells (18.80 ± 3.36 and 11.50 ± 4.42%) for both slow freezing and vitrification methods, respectively (Figure 18 C). However, the total cells were observed after preserved not apoptosis during cryopreservation for both methods.

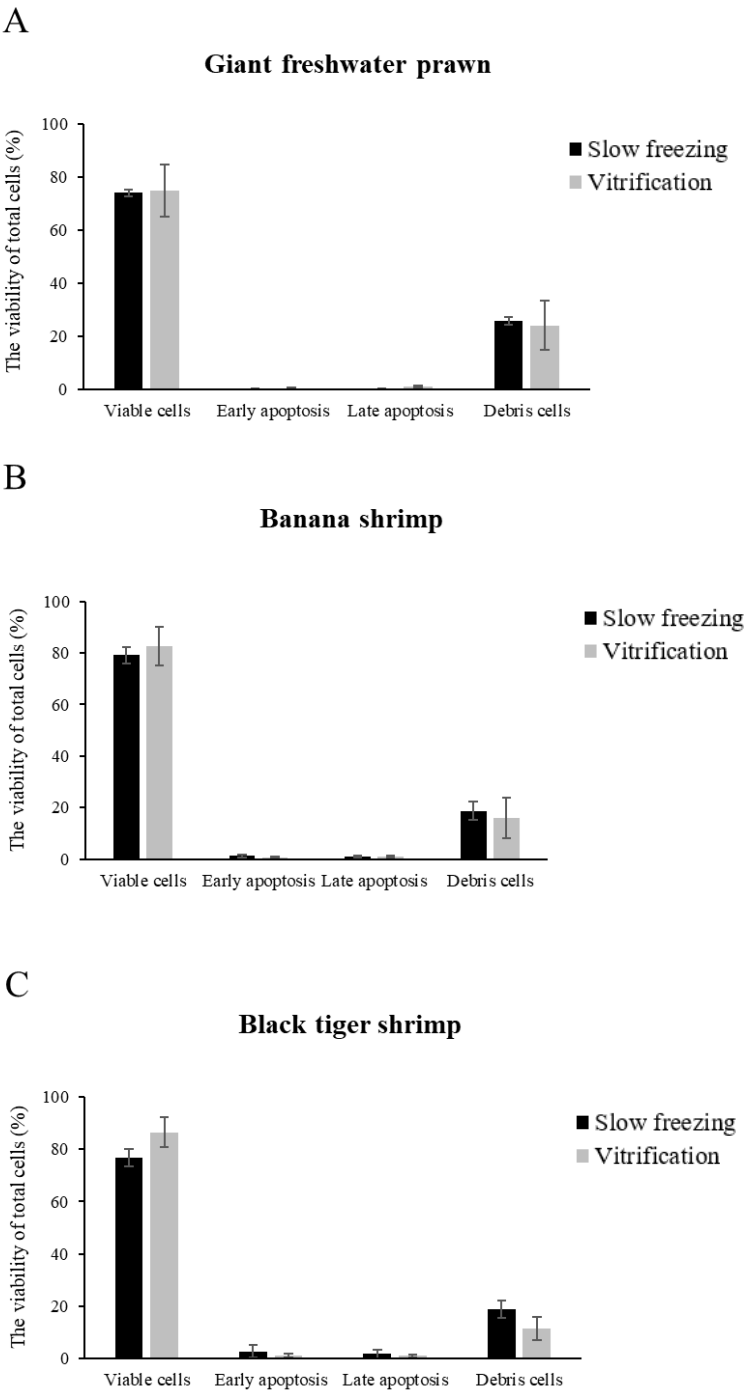


Figure 18. Flow cytometry of cryopreserved spermatogonia cells labeled with Annexin V and 7-AAD. Comparison of viability of total cells in giant freshwater prawn (A), banana shrimp (B) and black tiger shrimp (C) between slow freezing and vitrification methods for long-term cryopreservation (3 months). Each bar represents the mean ± SD (n=3).

CHAPTER 4

DISCUSSION

4.1 Identification of germ cells developmental stages in testes gonads of the giant freshwater prawn, banana shrimp and black tiger shrimp.

Spermatogonia cell have the ability to develop into functional gametes (sperm and eggs) and they are sufficiently small (10-µm diameter in medaka (Seki et al. 2017) and 8-10-µm diameter in giant freshwater prawn (Poljaroen et al. 2010)) and small enough to be cryopreserved. In order to preserve the spermatogonia cell (SG) of giant freshwater prawn, banana shrimp and black tiger shrimp for cryopreservation studies, it is important to identify and isolate spermatogonia cell population for preserving.

VASA is an important member of DEAD-box family. It is one of the important regulatory factors that determine the development of the reproductive system (Wang et al. 2012). It is a conserved gene whose expression is restricted to germ cells.

Therefore, in the present study, we focused on spermatogonia cell used VASA for identification spermatogonia cell in giant freshwater prawn, banana shrimp and black tiger shrimp for cryopreservation studies.

4.1.1 Identification of germ cells developmental stages in testes gonads of the giant freshwater prawn by immunohistochemical (IHC) analysis.

To identify the germ cells developmental stage for cryopreservation of giant freshwater prawn, identification of vasa protein in giant freshwater prawn was immunohistochemically stained on the section testes with anti-Vasa antibody. The signal of vasa protein was specifically detected in stages of spermatogonia cell (SG) but was not detected in stages of spermatocyte (SC)

and sperm (S), demonstrating that anti-Vasa antibody staining can be used as a powerful tool for investigating germ cell development stages in testes gonads of the giant freshwater prawn. Expression of vasa protein in spermatogonia cell of giant freshwater prawn testes is consistent with observation on Vasa protein expression in the Gibel Carp (Xu and Hong 2005), during spermatogenesis, the Gibel Carp Vasa is stained most intense in spermatogonia cell and disappears in sperm.

4.1.2 Expression analysis of vasa mRNA in banana shrimp and black tiger shrimp testes by RT-PCR and *in situ* hybridization (ISH)

Vasa gene is play an important role in spermatogenesis (Wang et al. 2012) and specifically expressed in the germ cell (Nakkrasae and Damrongphol 2007) that has been demonstrated in many organisms. The functions of *vasa* gene are vary depending on animal species. In *Drosophila*, mutations in the *vasa* gene cause a defect in the development of germline precursor cells (Tomancak et al. 1998); the *vasa* gene is necessary for the development of oocytes and spermatocytes during early stages of oogenesis and spermatogenesis (Styhler et al. 1998; Tomancak et al. 1998). In zebrafish, Yoon et al (1997) showed that *vasa* homolog transcripts were only found in PGCs, and that vasa RNA positive cells could be traced back to many cells at different stages of cleavage and vasa was specifically expressed in germ cells in frogs, mice, zebrafish, medaka, and rainbow trout. (Fujiwara et al. 1994, Komiya et al. 1994, Olsen et al. 1997, Shimomiya et al. 2000, Yoon et al. 1997; Yoshizaki et al. 2000). The vasa gene is thus an excellent candidate for using as a general molecular marker of primordial germ cell (PGC).

In situ hybridization, vasa-positive cells were detected in the testes of adult banana shrimp and black tiger shrimp. In adult testes, which contained mainly spermatogonia cell and spermatid, vasa-positive signals were specifically detected in spermatogonia cell, and no signals of vasa RNA were detected in spermatid. Expression of vasa mRNA in banana shrimp and black

tiger shrimp testes is consistent with previous studies in bluefin tuna (Nagasawa et al. 2009), vasa mRNA was thus predominantly localized in the type A spermatogonia cell, with weak expression observed in type B spermatogonia cell of bluefin tuna testes. Furthermore, no vasa mRNA was observed in spermatid, spermatozoa, or any of the gonadal somatic cells, which corresponding with tissue distribution patterns of *vasa* mRNA were analyzed in adult male of banana shrimp and black tiger shrimp tissues by RT-PCR. Therefore, we conclude that the cell identified in this study is the spermatogonia cell, which is primordial germ cell of banana shrimp and black tiger shrimp and used it for cryopreservation studies.

4.2 Cryopreservation experiment of testes

Cryopreservation method has been used to successfully preserve the spermatogonia cells in fish species, such as rainbow trout (Lee et al. 2013), Nile tilapia (Higaki et al. 2018) and common carp (Franěk et al. 2019); but not yet report mentioned successful cryopreservation of spermatogonia cell in shrimp. In this study, we have optimized factors for the cryopreservation experiment for giant freshwater prawn, black tiger shrimp and banana shrimp spermatogonia cell through slow freezing and vitrification methods of testes tissue. Because the cryopreservation method involved several factors that need to increase post-thaw survivability, also requires a type and concentration of cryoprotectant to prevent ice formation and thawing optimal temperature.

Previously, the effectiveness of DMSO for cryopreservation of sperm has been reported in various groups of animals, including tench (*Tinca tinca*) and goldfish (Marinović et al. 2017; Franěk et al. 2019), salmonids (Lee et al. 2013, Lee and Yoshizaki 2016; Franěk et al. 2019), cyprinids (Marinović et al. 2017, Linhartova et al., 2014; Franěk et al. 2019), shellfish (Gwo et al. 2002; Chao et al. 2009), and mud

crab (*Scylla serrata*) (Chao et al. 2009). In other studies, glycerol was found to be most effective for sperm cryopreservation of horseshoe crab, *Limulus polyphemus* (Behlmer and Brown 1984; Chao et al. 2009) and $MgCl_2$ has been used as a cryoprotectant for banana shrimp spermatophores (Memon et al. 2012). However, this study demonstrated that spermatogonia cell of giant freshwater prawn, black tiger shrimp and banana shrimp could be preserved from cryopreservation of testes. These findings reveal that giant freshwater prawn and banana shrimp germ cells can be preserved in DMSO and black tiger shrimp germ cells can be preserved in GLY. Interestingly, $MgCl_2$ could not protect germ cells in banana shrimp and black tiger shrimp during either slow freezing or vitrifcation, but contrasts with the result of viability and recovery in giant freshwater was 82.78 ± 4.56 and 62.41 ± 8.68%, respectively when stored in 10% of $MgCl_2$ with slow freezing method. These contrasting results can be explained by differences in the species. Thus, cryoprotection methods need to be optimized for each species.

Cell integrity is affected by cryoprotectant concentration. Because of the electrical characteristics of the cryoprotectant solution, a concentration that is too low may be insufficient to protect the cell against ice crystal formation, whereas a concentration that is too high may be hazardous to the cell membrane. Cryoprotectant concentrations that work best are between 5% and 15% (Wowk et al. 2000). Although giant freshwater prawn, banana shrimp, and black tiger shrimp germ cells survived at all concentrations of DMSO and GLY, cell recovery was dependent on the dosage during slow freezing and vitrification. Excessive doses of cryoprotectant have been found in studies to reduce ice crystal formation during vitrification; yet high amounts can harm cells by inducing metabolic and osmotic injuries. According to these studies, the most efficient concentration for vitrification is 10%.

In cryopreservation, the temperature at which the ice is thawed is critical. Warming frozen cells can cause recrystallization, cell hydration, and intracellular devitrification. Cell damage from these occurrences can be avoided with the right thawing temperature (Karlsson 2001). For giant freshwater prawn, optimal thawing temperatures are 30 °C (Akarasanon and Poolsanguan 2004) and for banana shrimp and black tiger shrimp spermatophores, optimal thawing temperatures are 27–30 °C and 30 °C, respectively (Memon et al. 2012; Nimrat et al. 2020; Vuthiphandchai et al.

2007). Lower thawing temperatures have been linked to improved sperm viability in studies. We used data from other species because there aren't many publications on germ cell cryopreservation in crustaceans. The germ cells of rainbow trout, common carp, and Siberian sturgeon have been preserved at temperatures of 10, 26, and 38 °C, respectively (Lee et al. 2013; Penika et al. 2016; Yoshikawa et al. 2018). As a result, we tested thawing temperatures of 10 and 27 °C and discovered that when the thawing temperature was lower, the recovery rates of shrimp germ cells increased.

For long-term survival of giant freshwater prawn, banana shrimp, and black tiger shrimp germ cells, vitrification was more successful than gradual freezing, according to our findings. The reason of effective of spermatogonia cell from vitrification method might be because that vitrification is a modern cryopreservation technique that combines an extremely rapid cooling rate for cells with an extremely high concentration of CPAs in cell suspension to lower the freezing temperature before abrupt cooling (Guven and Demirci 2012). Cell culture media containing a high concentration of CPAs underwent a rapid transformation from liquid to glass during the vitrification phase, minimizing cell harm (Zhang et al. 2018). Vitrification reduces the risk of mechanical damage to cells by preventing the development of ice crystals. Several studies conducted fish (Lujić et al. 2017, Marinović et al. 2018), bird and mammalian testicular (Liu et al. 2013) and ovarian tissue (Wang et al. 2008) indicated that when compared to the slow freeing process, vitrification has a number of advantages, including cost and time savings, as well as the use of less liquid nitrogen. Thus, vitrification method is useful for long-term storage of spermatogonia cell in giant freshwater prawn, banana shrimp and black tiger shrimp.

These findings indicate that cryopreservation can successfully preserve shrimp germ cells. Thus, the full developmental competence of theses cryopreserved spermatogonia cells need to be confirmed differentiation and proliferation ability of cryopreserved spermatogonia cells using in vivo and/or in vitro gametogenesis followed by artificial insemination and/or transplantation in future studies.

4.3 Flow cytometry

Apoptosis or the process of programmed cell death, is generally characterized by distinct morphological characteristics and energy-dependent biochemical mechanisms. Apoptosis is considered a vital component of various processes including normal cell turnover, proper development and functioning of the immune system, hormone-dependent atrophy, embryonic development, and chemical-induced cell death.

For cryopreservation experiment, the total cells after thawing from testes frozen in liquid nitrogen were determined apoptosis by flow cytometry analysis. It demonstrates that spermatogonia cell after preserve can viable and not apoptosis during cryopreservation. The results suggest that the method is well accepted and whether there is any possibility of further improvement remains to be studies in the future.

CHAPTER 5

CONCLUSION

This study examined an optimal factor for cryopreservation of giant freshwater prawn, banana shrimp and black tiger shrimp spermatogonia cell by slow freezing and vitrification methods.

For identification of spermatogonia cell in giant freshwater prawn, banana shrimp and black tiger shrimp, VASA is specifically expressed in the spermatogonia cell, which is germ cell. It is a potential molecular marker for study of spermatogonia cell cryopreservation.

For cryopreservation studies, 10% of DMSO was the best cryoprotectants for spermatogonia cell of giant freshwater prawn and banana shrimp and 10% of GLY was the best cryoprotectants for spermatogonia cell of black tiger shrimp and thawing temperature at 10 ℃ was suitable temperature for spermatogonia. Spermatogonia cell of shrimp can preserved in liquid nitrogen for long-term storage period up to 6 months and the total cells were observed after preserved not apoptosis during cryopreservation for both methods. This study showed that cryopreservation of spermatogonia cell in giant freshwater prawn, banana shrimp and black tiger shrimp can be successfully. Importantly, our study demonstrate that cryopreservation can be successfully performed without requiring no special or expensive equipment (freezing container and -80 ℃ freezer) by using vitrification method. Additionally, the recovery of spermatogonia cells preserved with vitrification method were significantly higher than of preserved with slow freezing method in all shrimps, thus vitrification is optimal method for long-term storage.

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APPENDIX

1. **Preparation of chemical stock solution for gel electrophoresis**

0.5 EDTA (pH8.0, 1000 ml)

Add 148 g of EDTA to 800 ml of DW, dissolved with magnetic stirrer. Adjust pH to 8.00 and fill up to 1000 ml with DW. Sterilize by autoclave at 121 °C for 15 min and keep at RT.

10X TAE buffer (Tris acetate EDTA buffer, 1000 ml)

Add 48.80 g of Tris-base, 20 ml of 0.5 EDTA and 11.42 ml of Glacial acetic to 500 ml of DW in beaker. Fill up to 1000 ml with DW, mix and sterilize by autoclave at 121 °C for 15 min and keep at RT.

2. **Preparation of chemical stock solution for cloning**

Luria-Bertaini (LB) broth (1000 ml)

Add 10 g of Tryptone, 5 g of Yeast extract powder and 5 g of NaCl to 500 ml of DW. Fill up to 1000 ml with DW, mix and sterilize by autoclave at 121 °C for 15 min, cooled and keep at 4 °C.

Luria-Bertaini (LB) agar (1000 ml)

Add 10 g of Tryptone, 5 g of Yeast extract powder, 5 g of NaCl and 15 g of agar to 500 ml of DW. Fill up to 1000 ml with DW, mix and sterilize by autoclave at 121 °C for 15 min, aliquots to plate and keep at 4 °C.

Ampicillin (80 mg/ml)

Sterilize DW by autoclave at 121 °C for 15 min, cool, add 80 g of Ampicillin sodium salt, mix and keep at 4 °C.

10% SDS (50 ml)

Add 5 g of SDS to DW, fill up to 50 ml with DW. Sterilize by autoclave at 121 °C for 15 min, cool and keep at RT.

1 N NaOH (50 ml)

Add 2 g of NaOH to DW, fill up to 50 ml with DW. Sterilize by autoclave at 121 °C for 15 min, cool and keep at RT.

Solutions for Plasmid extraction

Solution I (50 ml)

Add 015 g of Tris to 30 ml of DW and adjust pH to 8.0 with HCL. Add 0.45 g of glucose and 0.19 g of EDTA and fill up to 50 ml with DW. Sterilize by autoclave at 121 °C for 15 min, cool and keep at 4 °C.

Solution II (50 ml) (Prepare before use)

Add 100 µl of 10% SDS, 200 µl of 1N NaOH and fill up to 1 ml with DI.

Solution III (50 ml)

Add 14.72 g of Potassium acetate to 30 ml of DI, add 5.1 ml of Glacial acetic acid and fill up to 1 ml with DI. Sterilize by autoclave at 121 °C for 15 min, cool and keep at RT.

3. Preparation fixative solution

Bouin's solution (Prepare before use)

Add 1 ml of Acetic acid, 5 ml of Formalin and 15 ml of Picric acid to tube, mix and keep at 4 °C.

8% Paraformaldehyde (PFA) stock (15 ml)

Add 3.24 of 37% PFA to DEPC water, mix and keep at RT.

4. Preparation chemical stock solution for immunohistochemical analysis

PBS buffer (pH 7.4, 100ml)

Add 0.144 g of Na_2HPO_4, 0.02 g of KH_2PO_4 , 0.8 g of NaCl and 0.02 g of KCL to 90 ml of DW. Adjust pH to 7.4 with NaCl and adjust the final volume to 100 ml with DW. Sterilize by autoclave at 121 °C for 15 min, cooled and keep at RT.

10X TBS (pH 7.5, 500 ml)

Add 40 g of NaCl, 1 g of KCL and 15 g of Tris to 400 ml of DW. Adjust pH to 7.5 with HCl and fill up to 500 ml with DW. Sterilize by autoclave at 121 °C for 15 min, cool and keep at RT.

1X TBST (500 ml)

Add 50 ml of 10X TBS and 0.5 ml of Tween to 450 of DW, mix and keep at RT.

5. Preparation chemical stock solution for *in situ* hybridization

DEPC water (2000 ml)

Mix 2 ml of DEPC with 2000 ml of DI in Duran. Incubate for ~1-2 h at RT with magnetic stirrer. Sterilize by autoclave at 121 °C for 15 min, cool and keep at RT.

Proteinase K buffer (Prepare before use)

Mix 7.5 µl of Proteinase K stock solution (20 mg/ml) with 50 ml of TBST in Duran and incubate in 37 °C before use.

Acetylation buffer (pH 8.0, 500ml)

Add 6.1 g of Tris to 450 ml of DEPC water and dissolve. Adjust pH to 8.0 and fill up to 500 ml with DEPC water. Sterilize by autoclave at 121 °C for 15 min, cool and keep at RT.

Moisting buffer (50 ml)

Add 25 ml of Formamide, 12.5 ml of 20X SSC (pH 4.5) and 12.5 ml of DEPC water to Duran, mix and keep at RT.

20X SSC (pH 4.5, 500 ml)

Add 87.6 g of NaCl with 44.1 g of Trisodium citrate dihydrate to 400 ml of DEPC water in Duran. Adjust pH with 10N NaOH, fill up to 500 ml with DEPC water. Sterilize by autoclave at 121 ℃ for 15 min, cool and keep at RT.

Maleic buffer (500 ml)

Add 5.5 g of Maleic acid and 4.385 g of NaCl to 400 ml of DEPC water in Duran. Adjust pH with 10N NaOH and fill up to 500 ml with DEPC water. Sterilize by autoclave at 121 ℃ for 15 min, cool and keep at RT.

10% Blocking reagent (200 ml)

Dissolve 20 g of Blocking reagent with Maleic buffer, warm in the microwave or hot water and shaking. After dissolve completely fill up to 200 ml with Maleic buffer and homogenize by shaking. Sterilize by autoclave at 121 °C for 15 min, cool and keep at -20 °C.

Blocking solution (150 ml)

Mix 30 ml of 10% Blocking reagent with 120 ml 0f TBST in Duran and keep at -20 °C.

5 M NaCl (500 ml)

Dissolve 146.1 g of NaCl with 400 ml DEPC water by magnetic stirrer and fill up to 500 ml with DEPC water. Sterilize by autoclave at 121 °C for 15 min, cool and keep at RT.

2 M Tris HCL (pH 8.0) (1000 ml)

Dissolve 242.2 g of Tris base with 800 ml DEPC water by magnetic stirrer, adjust pH to 8.0 with HCL and fill up to 1000 ml with DEPC water. Sterilize by autoclave at 121 °C for 15 min, cool and keep at RT.

0.5 M EDTA (pH 8.0) (100 ml)

Dissolve 18.61 g of EDTA with 80 ml DEPC water by magnetic stirrer, adjust pH to 8.0 with HCL and fill up to 100 ml with DEPC water. Sterilize by autoclave at 121 °C for 15 min, cool and keep at RT.

1 M $MgCl_2$ (100 ml)

Dissolve 20.3 g of $MgCl_2 \cdot 6H_2O$ with 80 ml DEPC water by magnetic stirrer and fill up to 100 ml with DEPC water. Sterilize by autoclave at 121 °C for 15 min, cool and keep at RT.

2 M Tris HCL (pH 9.5)

Dissolve 242.2 g of Tris base with 800 ml DEPC water by magnetic stirrer, adjust pH to 9.5 with HCL and fill up to 1000 ml with DEPC water. Sterilize by autoclave at 121 °C for 15 min, cool and keep at RT.

NTE buffer (Prepare before used)

Mix 100 ml of 5M NaCl, 5 ml of 2M Tris HCL (pH 8.0) and 2 ml of 0.5M EDTA (pH 8.0) in Duran.

RNAse buffer (Prepare before used)

Add 50 µl of RNAse stock (10 mg/ml) to 50 ml of NTE buffer and mix.

NTMT buffer (Prepare before used)

Add 3 ml of 5M NaCl, 7.5 ml of 1M $MgCl_2$, 7.5 ml of 2M Tris HCL (pH 9.5) and 150 µl of Tween to Duran. Fill up to 150 ml with DEPC water.

6. Preparation chemical stock solution for cryopreservation

Leibovitz's L-15 medium (pH 7.8, 100 ml)

Dissolve 1.37 g of Leibovitz's L-15 and D-Glucose in 88 ml of DI with magnetic stirrer for 4 hours at room temperature. Adjust pH to 7.8 and add 10 ml of FBS and 1 ml of anti-anti. Then filtered through a 0.45 µm-pore nylon screen into tube and keep at 4 °C.

1 M trehalose

Dissolve 3.7833 g of Trehalose with 10 ml of DI by shaking and keep at 4 °C.

1 M $CaCl_2 \cdot 2H_2O$

Dissolve 1.4702 g of $CaCl_2 \cdot 2H_2O$ with 10 ml of DI by shaking and keep at 4 °C.

1 M $MgCl_2 \cdot 2H_2O$

Dissolve 2.0330 g of $MgCl_2 \cdot 2H_2O$ with 10 ml of DI by shaking and keep at 4 °C.

10X Extender stock

Add 4.61 g of HEPES, 7.68 g of NaCl, 0.19 g of KCL, 1.1 g of KH_2PO_4, 0.19 g of Na_2HPO_4 and 0.14 g of Sodium pyruvate to DW and fill up to 70 ml with DW and mix. Sterilize by autoclave at 121 °C for 15 min, cool and keep at 4 °C.

2X Extender (Prepare before use)

Add 294 µl of 10X Extender, 3.9 µl of $CaCl_2 \cdot 2H_2O$, 2.1 µl of $MgCl_2 \cdot 2H_2O$, and fill up to 1.5 ml of DW. Mix and incubate on ice.

3.3X Extender (Prepare before use)

Add 485.1 µl of 10X Extender, 6.435 µl of $CaCl_2 \cdot 2H_2O$, 3.465 µl of $MgCl_2 \cdot 2H_2O$, and fill up to 1.5 ml with DW. Mix and incubate on ice.

Cryomedium (Prepare before use)

Add 176 µl of 2X Extender, 50 µl of 1M Trehalose, Cryoprotectant, 50 µl of BSA and fill up to 0.5 ml with DW. Mix and incubate on ice for 10 min before use.

Vitrification solution

Add 300 µl of 3.3X Extender, 200 µl of FBS, 200 µl of 1M Trehalose, Cryoprotectant, 150 µl of Ethylene glycol and fill up to 1 ml with DW. Mix and incubate on ice for 10 min before use.

7. Preparation chemical stock solution for dissociation cell

DNase I stock (15 u/ µl**)**

Mix 1.5 µl of DNase I with 998.5 µl of DI by shaking. Keep at -20 °C.

Collagenase H (40 mg/ml)

Mix 40 mg of Collagenase H with 1 ml of DI by shaking. Keep at -20°C.

Dispase II (33.3 mg/ml)

Mix 33.3 mg of Dispase II with 1 ml of DI by shaking. Keep at -20 °C.

Dissociation cell solution

Add 435 µl of Leibovitz's L-15 medium, 25 µl of Dispase II, 25 µl of Collagenase H, 15 µl of DNase I and mix by pipetting.

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