DEVELOPMENT OF CRYOPRESERVATION OF TESTES IN THE ASIAN SEA BASS (*LATES CALCARIFER*)

Somkiat Sreebun

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การพัฒนาการแช่แข็งอัณฑะของปลากะพงขาว (*LATES CALCARIFER*)

นายสมเกียรติ ศรีบุญ

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for a Master's degree.

Thesis Examining Committee

(Assoc. Prof. Dr. Amonrat Molee)
Chairperson

(Assoc. Prof. Dr. Surintorn Boonanuntanasarn)
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สมเกียรติ ศรีบุญ: การพัฒนาการแช่แข็งอัณฑะของปลากะพงขาว *(Lates calcarifer)* (DEVELOPMENT OF CRYOPRESERVATION OF TESTIS IN THE ASIAN SEA BASS *(LATES CALCARIFER)*) อาจารย์ที่ปรึกษา: รองศาสตราจารย์ ดร. สุรินทร บุญอนันธนสาร, 66 หน้า.

คำสำคัญ: ปลากะพงขาว/การแช่แข็ง/การปลูกถ่ายเซลล์สืบพันธุ์ *Lates calcarifer*

การวางแผนในการอนุรักษ์เป็นวิธีการหนึ่งในการเก็บรักษาทรัพยากรพันธุกรรมในปลาต่าง ๆ ซึ่งจะสามารถนำกลับมาใช้ ผลิตหรือผสมได้ในระยะยาว โดยทั่วไปวิธีการที่ใช้ในการอนุรักษ์สามารถทำได้โดยการรวมกันระหว่างวิธีการเก็บรักษาด้วยกระบวนการการแช่แข็งซึ่งเป็นการจัดเก็บเนื้อเยื่อและ (หรือ) เซลล์ และวิธีการปลูกถ่าย ปลากะพงขาว (*Lates calcarifer*) เป็นปลาที่มีความสำคัญทางเศรษฐกิจในด้านการค้าในกลุ่มของปลาทะเล การอนุรักษ์ทรัพยากรทางพันธุกรรมของปลากะพงขาวเพื่อใช้ในการเพาะเลี้ยงสัตว์น้ำในอนาคต ดังนั้น วัตถุประสงค์ของการศึกษาในครั้งนี้เพื่อศึกษาสภาวะที่เหมาะสมต่อวิธีการเก็บรักษาอัณฑะแบบแช่แข็งของปลากะพงขาว และการปลูกถ่ายเซลล์สืบพันธุ์ที่ผ่านการแช่แข็งของอัณฑะปลาแช่แข็งในปลาผู้รับ

การหาวิธีการแช่แข็งโดยการแช่แข็งแบบช้าที่เหมาะสมประกอบด้วยการหา cryomedium และอุณหภูมิที่ใช้ในการละลายหลังการแช่แข็งที่เหมาะสม โดยสาร ในการศึกษาครั้งนี้ extender สามชนิดคือ Mounib NAM หรือ L-15 ทำงานร่วมกับ cryoprotectants สามชนิดคือ DMSO EG หรือ PG ในขั้นตอนถัดไป หาความเข้มข้นของ cryoprotectants ที่เหมาะสมที่ความเข้มข้น 7.5% 10% 12.5% หรือ 15.0% (v/v). การทดลองถัดไปเมื่อได้สภาวะแช่แข็งแล้ว ทำการศึกษาอุณหภูมิและเวลาที่เหมาะสมในการละลายหลังจากการแช่แข็งโดยอุณหภูมิที่ใช้ในการทดลองมี 2 อุณหภูมิ ได้แก่ 4 และ 10 องศาเซลเซียส ร่วมกับระยะเวลา 2 เวลา คือ 8 และ 10 นาที ในการศึกษาครั้งนี้ เซลล์ spermatogonia สามารถระบุได้จากสัณฐานวิทยา คือ มีขนาด 10 ไมโคเมตรและมีนิวเคลียสขนาดใหญ่ การย้อมสีด้วย Trypan blue เพื่อประเมินเปอร์เซ็นต์ของอัณฑะหลังการแช่แข็งเมื่อเทียบกับอัณฑะสด เพื่อตรวจสอบอัตราการมีชีวิตของเซลล์ spermatogonia ที่เก็บรักษาด้วยการแช่แข็ง การวิเคราะห์ flow cytometric ด้วย ฟลูออเรสซินไดอะซิเตต (FDA) และ โพรพิเดียมไอโอไดด์ (PI) นอกจากนี้ การวิเคราะห์ apoptosis โดยใช้ FITC-Annexin V and PI ในการย้อมสี และเพื่อยืนยันการคุณสมบัติของการเป็นเซลล์สืบพันธุ์ การวิเคราะห์ in situ hybridizationด้วย vasa antisense RNA. ต่อมาการตรวจสอบเซลล์ testicular ที่ผ่านการเก็บรักษาด้วยวิธีการแช่แข็งยังสามารถปลูกถ่ายได้หรือไม่ โดยอัณฑะสดและอัณฑะที่เก็บรักษาด้วยการแช่แข็งถูกนำมาย่อยเซลล์ และนำเซลล์ฉีดเข้าในช่องท้องของปลาผู้รับ ผลการศึกษาพบว่า cryomedium ประกอบด้วย L-15 และ DMSO ที่ความเข้มข้น 10% ให้ผลการรอดตายของเซลล์ spermatogonia เมื่ออัณฑะผ่านการแช่แข็งเท่ากับ 78.16%

+0.98% เมื่อเทียบกับอัณฑะสดที่ไม่ผ่านการแช่แข็งเท่ากับ 88.08%+0.58% การละลายอัณฑะแช่แข็งที่อุณหภูมิ 10 องศาเซลเซียสที่ระยะเวลา 8 นาที มีอัตราการรอดตายสูง ในการทดลองถัดไป การย้อมสีด้วย FDA/PI อัตรารอดระหว่างเซลล์สด (81.63±0.92%) และเซลล์ที่ไม่ผ่าการแช่แข็ง (81.06 ±1.11%) ไม่แตกต่างกัน ในการวิเคราะห์ Apoptosis พบว่าการเกิด apoptotic ในอัณฑะที่ผ่านการแช่แข็งมีอัตราการเกิดท่าสูงกว่าเมื่อเปรียบเทียบกับอัณฑะสด แสดงให้เห็นว่ากระบวนการหลังการละลายทำให้เกิดการตายของเซลล์เมื่อเปรียบเทียบกับอัณฑะสด การสังเกตผลที่เกิดขึ้นอาจบ่งชี้ว่า apoptosis เป็นกระบวนการศึกษาที่แบ่งเป็นระยะของการตายของเซลล์ที่เกิดขึ้นในการเก็บรักษาด้วยการแช่แข็งของอัณฑะ ในการใช้ in situ hybridization ทั้งเซลล์สดและเซลล์แช่แข็งพบแสดงออกของยีน vasa แสดงว่า spermatogonia ที่ผ่านการแช่แข็งยังมีคุณสมบัติของเซลล์ต้นกำเนิด และในการปลูกถ่ายเซลล์สืบพันธุ์พบว่าเซลล์ testicular ทั้งเซลล์สดและเซลล์แช่แข็ง สามารถเคลื่อนที่และรวมตัวกันใน genital ridge ของปลาผู้รับเมื่อนำมาเชื่อมโยงกัน วิธีการแช่แข็งของอัณฑะของปลากะพงขาว และการปลูกถ่ายเซลล์สืบพันธ์ที่ผ่านการแช่แข็งแล้ว ได้ข้อเสนอว่า การทำงานร่วมกันของการแช่แข็งอวัยวะสืบพันธ์ ร่วมกับ การปลูกถ่ายเซลล์สืบพันธ์จะทำให้กลายเป็นเครื่องมือที่ใช้ในการเก็บรักษาปลากะพงขาวได้

สาขาวิชาเทคโนโลยีและนวัตกรรมทางสัตว์ ลายมือชื่อนักศึกษา สมเกียง สยุมพู

ปีการศึกษา 2565 ลายมือชื่ออาจารย์ที่ปรึกษา ศิรินทร์

SOMKIAT SREEBUN : DEVELOPMENT OF CRYOPRESERVATION OF TESTIS IN THE ASIAN SEA BASS (*LATES CALCARIFER*). THESIS ADVISOR : ASSOC. PROF. SURINTORN BOONANUNTANASAEN, Ph.D., 66 PP.

Keyword: ASIAN SEA BASS/*LATES CALCARIFER*/CRYOPRESERVATION/GERM CELL TRANSPLANTATION

Preservation strategy has provided a tool to maintain genetic resources in fish which could be restored for long-term uses. In general, a preservation tool could be achieved by combination of cryopreservation which is a process for frozen storage of tissues and/or cells and transplantation. The Asian sea bass (*Lates calcarifer*) has been economically important in the trade of marine fish which is needed to preserve their genetic resources for use in aquaculture in the future. Therefore, the objectives of this study are to determine suitable cryopreservation methods of whole testes in the Asian sea bass and to transplant the cryopreserved testicular cell in allogenic recipient.

To optimize the cryopreservation method using a slow freezing process, suitable cryomedium and thawing processes were determined. This study investigated cryomedium containing three types of extenders (Mounib, NAM or L-1) and three types of cryoprotectants (DMSO, EG, or PG). Next, two suitable cryoprotectants were determined for their optimal concentration including 7.5%, 10 %, 12.5%, or 15.0% (v/v). Subsequently, the best cryomedium was used to explore thawing conditions including temperatures at 4°C or 10°C and times for 4, 8, or 10 min. Through this study, spermatogonia-like cell was identified by its morphological characteristic including 10 µm diameter and large nucleus. Trypan blue staining was used to evaluate the percentage of spermatogonia of cryopreserved testes compared with fresh testes. To validate the viability rate of cryopreserved spermatogonia cell, flow cytometric analysis with fluorescein diacetate (FDA) and propidium iodide (PI) were performed. In addition, apoptosis analysis was carried out using FITC-Annexin V and PI staining. To confirm the function of germ cell, in situ hybridization analysis was also performed using *vasa* antisense RNA. Moreover, to determine whether the cryopreserved testicular cell could exhibit transplantability, fresh and cryopreserved testes were dissociated and intraperitoneally microinjected

into allogenic recipient larvae. Our results showed that suitable cryomedium containing L-15 and 10% DMSO at 10% could gave the viability rate of spermatogonia obtained from cryopreserved testes (78.16%±0.98%) comparing with that obtained from fresh testes (88.08%±0.58%). Thawing conditions at 10°C for 8 min resulted in the highest viability rate. Using FDA/PI staining, a similar viability rate between fresh (81.63±0.92%) and cryopreserved cells (81.06±1.11%) was detectable. Apoptosis analysis showed early apoptotic cell in cryopreserved testes was significantly higher than that obtained from the fresh testes, demonstrating that the post-thaw process induced apoptosis compared to fresh testes. This observation could suggest that apoptosis, which is a process of programmed cell death, occurred in cryopreservation of whole testes. Using in situ hybridization, spermatogonial cell obtained from both fresh and cryopreserved testes showed positive *vasa* expression, suggesting its spermatogonia stem cell characteristic. Our results showed that testicular cells obtained from cryopreserved testes could migrate and incorporate into the genital ridge of recipient fry. Taken together, the cryopreservation method for whole testes of the Asian sea bass was demonstrated, and the cryopreserved testicular cell exhibit transplantability, suggesting that cryopreservation when combined with transplantation would make it possible to become preservation tool.

School of Animal Technology and Innovation Student's Signature Somkiat Sreetan
Academic Year 2022 Advisor's Signature

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LIST OF ABBREVIATIONS

ANOVA	=	Analysis of variance
ASG	=	Type A spermatogonia
BW	=	Body weight
DMSO	=	Dimethyl Sulfoxide
dpf	=	Days post fertilization
dph	=	Days post hatching
dpt	=	Days post transplantation
EG	=	Ethylene glycol
FBS	=	Fetal bovine serum
FDA	=	fluorescein diacetate
GCT	=	Germ cell transplantation
GLY	=	glycerol
GSI	=	Gonadosomatic index
H&E	=	Hematoxylin and eosin
Kg	=	kilogram
L	=	liter
L-15	=	L-15 Medium (Leibovitz)
m	=	mater
M	=	Molarity
Me2SO	=	Dimethyl Sulfoxide
mg	=	milligrams
mRNA	=	Messenger RNA
NAM	=	Non-Activating Medium
OG	=	Oogonia
PBS	=	Phosphate Buffered Saline
PG	=	propylene glycol
DMA	=	dimethyl acetamide
PGC	=	Primordial germ cell

LIST OF ABBREVIATIONS (Continued)

PI	=	propidium iodide
PO	=	Previtellogenic oocyte
ppt	=	part per thousand
PSC	=	Primary spermatocyte
RBC	=	Red blood cell
RT	=	rainbow trout
SD	=	standard deviation
SG	=	Spermatogonia cells
SPC	=	Secondary spermatocyte
SPSS	=	Statistical Package for Social Sciences
SZ	=	Spermatozoa
TB	=	trypan blue
u/ml	=	Unit/milliliter
μm	=	Micromete

CHAPTER I
INTRODUCTION

1.1 Introduction

The Asian sea bass is commercially important seawater fish, particularly in Southeast Asia. Global aquaculture production of the Asian sea bass has been increased. The Asian Sea bass is high market value, and its demand has increased every year. In Thailand, the Asian sea bass products have been obtained from both capture and aquaculture, and the amount of aquaculture production has been much more than that obtained from capture. Since the Asian sea bass production has been cultured and provided as the main source of seawater fish for both domestic consumption and export, research, and development for preservation of diversity of genetic resources are needed to develop. Preservation of germ cell of the Asian sea bass would enable the risk management of the Asian sea bass farming. In addition, it would be the useful technology to maintain the diversity of genetic resources. Indeed, preservation tool can be achieved by farming of the Asian sea bass as many as possible to maintain diversity of genetic resources. However, the cost of the farming fish is high, and it is also labor intensive. Therefore, development of farming independent preservation of genetic resources such as application of cryopreservation of germ cell would provide as effective tool.

Cryopreservation is the process to preserve intact living cells and tissues using process of cooling and storing cells (Amann and Pickett., 1987; Royer et al., 1996; Lin et al., 1999). Indeed, cryopreservation of germ cell such as sperm has been developed in a number of fish, particularly in the Asian sea bass (Leung, 1987; Palmer, 1993; Zilli et al., 2003). The cryopreservation of sperm technology enables preservation strategy to maintain high number of germ cell which is able to inherit its genetic material through their offspring. Recently, cryopreservation of testis which contains undifferentiated germ cell has been developed. Germ cell transplantation is a technique for transferring of undifferentiated germ cell from doner fish into the

gonad of recipient fish. Transplanted germ cell could be incorporated in the recipient gonad to produce functional gametes. Therefore, germ transplantation technology would be able to become surrogate broodstock to produce donor-derived offspring. Combination of cryopreservation of testis and transplantation was demonstrated in trout, and it was showed to produce donor-derived offspring (Lee et al., 2013). Therefore, development of cryopreservation of testis and germ cell transplantation in the Asian sea bass would provide as a useful tool for farming independent preservation of genetic resources for sustainable production of the Asian sea bass.

1.2 Objective

In order to develop preservation of genetic resources in the Asian sea bass for future use, the cryopreservation of testis methodology will be studied in the Asian sea bass. The specific objectives of this study are as following:

1.2.1 To determine cryomedium containing suitable extender and cryoprotectants and thawing condition for cryopreservation of testis in the Asian sea bass**.**

1.2.2 To develop germ cell transplantation using frozen testicular cell in the Asian sea bass.

1.3 hypotheses

1.3.1 Suitable cryopreservation methodology including cryomedium (cryomedium: extender and cryoprotectant) and thawing condition for testis would enable high viability of spermatogonia in the Asian sea bass.

1.3.2 The frozen testis obtained from suitable cryopreservation method could be used for germ cell transplantation in the Asian sea bass.

1.4 Scope of study

Thesis were study about cryopreservation of whole testis of Asian seabass weight 300-500 gram used difference extender, cryoprotectant, freezing rate and thawing rate for preserve testis.

1.5 Expected outcome

The suitable cryopreservation methodology would enable to preserve diversity of genetic resources in the Asian sea bass which will be provided for maintaining the diversity for aquaculture. In addition, the preservation of genetic resources would be useful for conservation aspect. Combination of the cryopreservation and germ cell transplantation in the Asian sea bass has potential to be a powerful tool to generate surrogate broodstock to produce offspring for future use.

1.6 Keyword

The Asian sea bass (*Lates calcarifer*), cryopreservation, germ cell transplantation.

CHAPTER II

LITERATURE REVIEWS

2.1 Asian sea bass

The Asian sea bass (Table 2.1.; Figure 2.1) has body elongated, compressed, with deep caudal peduncle. Its mouth is large, slightly oblique, and upper jaw reaching to behind eye. It has villiform teeth. Its dorsal fin contains 7 to 9 spines and 10 to 11 soft rays. It has short and rounded pectoral fins. The anal fin is round containing three spines and 7–8 soft rays. The caudal fin is also rounded. The scale is large and ctenoid. The Asian sea bass widely distributed in tropical and sub-tropical areas of the Western and Central Pacific and Indian ocean (between longitude 50°E-160°W latitude 24°N-25°S). It has been reported to found throughout the northern part of Asia, southward to Queensland (Australia), westward to East Africa. Found in coastal waters, estuaries, and lagoons. Usually occurs at depths of 10 to 40m. (Mathew, 2009).

Table 2.1 Taxonomy of the Asian sea bass.

Phylum	Chordata
Class	Pisces
Order	Percomorphi
Family	Centropomidae
Genus	*Lates*
Species	*Lates calcarifer*

Reference: Bloch (1970).

Figure 2.1 Asian sea bass (*Lates calcarifer*).

The Asian sea bass or barramundi (*L. calcarifer*) has been an economically important in Asian. The Asian sea bass has been obtained from capture and mariculture. In coastal aquaculture, the Asian sea bass has been produced in Australia, Singapore, Saudi Arabia, Malaysia, India, Indonesia, Vietnam, Israel, Thailand, the United States, Poland, and the United Kingdom. In Thailand, the total production of Asian sea bass has come from marine fisheries and coastal aquaculture. In 2017, Table 2.2 showed that the Asian sea bass was mainly obtained from coastal aquaculture. The Asian sea bass was obtained from the marine capture fisheries only 14 ton, and the main the Asian sea bass product was caught from the gulf of Thailand. Indeed, coastal fish culture in Thailand has mainly been the Asian sea bass and grouper (Figure 2.2A.). There were two types of the Asian sea bass culture, and the most type of the Asian sea bass culture were pond culture. (Figure 2.2B.). In Thailand, the Asian sea bass culture has been taken in coastal provinces, and the highest production the Asian sea bass was observed in Chachoengsao (Figure 2.3). Although the Asian sea bass has been the most economically important in marine fish culture, there have been limited information about the Asian sea bass genetic resources, genetic diversity and genetic improvement. Particularly, preservation of diversity of genetic resources is required in order to develop sustainable the Asian sea bass production in Thailand.

Table 2.2 Statistics on the Asian sea bass (*L. calcarifer*) production in 2017.

Items	
Total amount from coastal aquaculture	20,454 ton
Total amount from marine capture fisheries	14 ton
- Gulf of Thailand	10 ton
- Indian Ocean	4 ton
Value of the Asian sea bass from marine capture fisheries	2,023,000 Baht
- Gulf of Thailand	1,448,000 Baht
- Indian Ocean	575,000 Baht

Reference: (Department of fisheries, 2019).

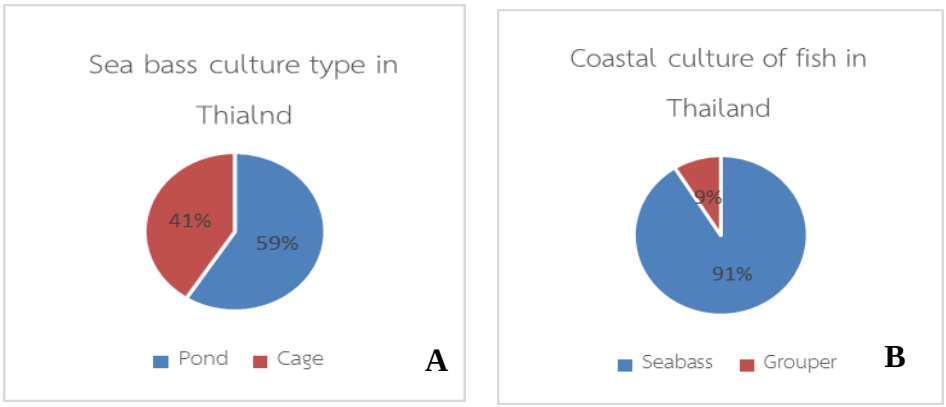

Figure 2.2 The Asian sea bass culture type in Thailand (A). Coastal culture of fish in Thailand (B).

Reference: Department of fisheries (2019).

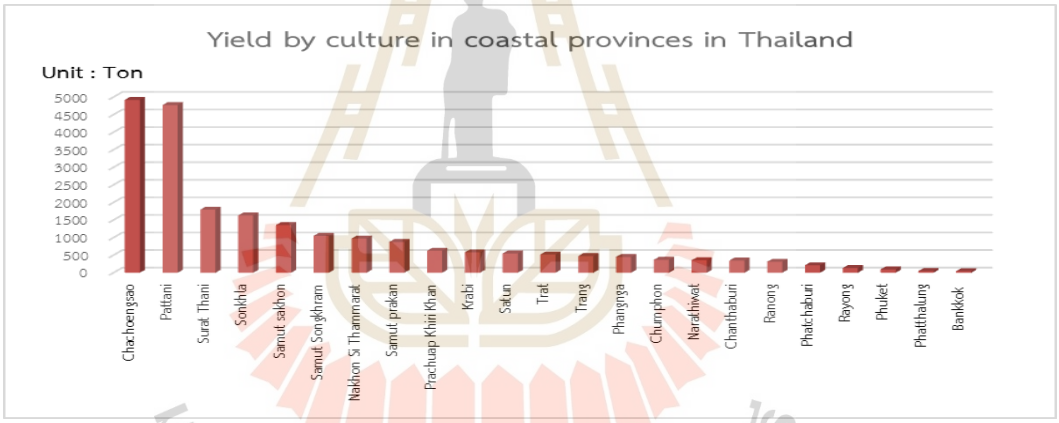

Figure 2.3 Yield of Sea bass by culture in coastal province (Unit: ton).

Reference: Department of fisheries (2019).

Development of full-cycle aquaculture is needed for sustainable aquaculture. Full-cycle fish culture includes producing of fish embryo, culturing artificially hatched larvae to adult fish, collecting some of adult and using as broodstock for producing of fish seed to create subsequent generations. Indeed, full-cycle aquaculture has been developed in aquaculture-related fish including the Asian sea bass. In addition, diversity of genetic resources in fish population stocks is required to prevent the inbreeding depression. Moreover, genetic improvement of fish would enable

increased in productivity. These diversities of genetic resources and new bred of fish with genetic improvement need to be preserved in order to ensure the safety of irreplaceable of valuable genetic resources. The preservation of genomes resources would facilitate broodstock management and prevent the risk of the loss of genetic diversity in fish population. Cryopreservation could be used as a powerful to preserve diversity of genetic resources and desired genetic traits for the long-term use of fish population which would be able to supply fish seed in future.

2.2 Cryopreservation

Cryopreservation is the process to preserve intact living cells and tissues using process of cooling and storing cells, tissues and/or organs. The processes of cooling and storing cells/tissues at a temperature below the freezing point -196°C allow high survival rates of the cells and/or tissues upon thawing process (Pegg, 2007). The cryopreservation technology has been important and benefit for preserved stem cells, tissues and/or embryo for investigation and/or use in future. Several factors are needed to investigate for development of cryopreservation including extenders, cryoprotectants, freezing conditions and thawing processes (Vuthiphandchai et al., 2009). The Cryomedium is the mixture of extender and cryoprotectant to prevent cell damage of freezing and thawing processes (Brian, 2007). Indeed, these cryomedium could be contained several functional ingredients such as antioxidant and energy providing substances (Lee et al., 2014). The freezing processes for decreasing temperature and thawing condition for increasing temperature have effects of cell viability (Sansone et al., 2002). To date, in fish, cryopreservation has been developed in sperm, primordial germ cell (PGC), spermatogonia, oogonia, and embryo (Betsy and Kumar, 2020). In sea bass, cryopreservation of sperm was developed (Martínez-Páramo et al., 2012.

2.2.1 Extender

Extender medium is basically the solution of balanced salts (Agarwal, 2011). It ideally is isotonic and contains buffering capacity with the cellular fluid of target species. It occasionally contains nutrients, sources of energy, antioxidants and antibacterial. For frozen sperm, extender is generally used for dilution of sperm

number and inhibit the activation of sperm or movement of the sperm (Muchlisin, 2004). The composition of extender is different according to fish species. Generally, formulation of extender varies to contain different salt, organic and inorganic components which is designed according to appropriate osmolality and pH (Samorn, 2007). For example, in the freshwater fish, the osmolality was generally 280-300 mOsm/Kg, and in the seawater fish, the osmolality was 200-300 mOsm/Kg (Wayman and Tiersch., 2000). The blood pH of fish was in range of 7.7-8.0 (Borinskaya, 2017). Some extenders that were revealed to be used for cryopreservation of the Asian sea bass included NAM (non-activating mineral medium), Mounib's solution and ringer solution (Table 2.3.). For gonadal tissue cryopreservation, several extenders were used for cryopreservation of testis and ovary (Table 2.4).

2.2.2 Cryoprotectant

During freezing process, freezing could cause damage to frozen cell. Forming of ice crystal could damage cellular shape. In addition, the change in solute concentration and osmotic of residual unfrozen water could damage frozen cell/tissues (Wowk, 2007). Therefore, cryoprotectants are required to add to protect cell damage by these effects. Cryoprotectants are chemicals that are used to protect cells from damage due to the formation of ice crystals during freezing and thawing (Muchlisin, 2004; Tiersch, 2006). Cryoprotectants have ability to dissolve in water and lower the melting point of water. Indeed, cryoprotectants enable cellular dehydration and shrinkage. Cryoprotectants decrease the dehydration damage during the process that water pass the cell to form ice crystal in the surrounding solution. There are two types of cryoprotectants including permeating cryoprotectants and non-permeating cryoprotectants. The permeating cryoprotectant can penetrate cell membrane. The molecular mass of permeable cryoprotectants is typically less than 100 Daltons (Muchlisin, 2004; Tiersch, 2006; Agarwal, 2011). There have been reported for a number of cryoprotectant use in cryopreservation of cell and tissue in fish including dimethyl sulphoxide, ethanol, ethylene glycol, glycerol, etc. Indeed, several cryoprotectants are toxic to cells (Muchlisin, 2004; Agarwal, 2011). Therefore, the optimum use of cryoprotectants is a balance between protection of cell damage and toxicity. The use of cryoprotectants need to investigate types of cryoprotectants and their optimal concentration (Wowk, 2007; Agarwal, 2011). Non-permeating

cryoprotectants such as sugars and polymers remain outside during the process of freezing which maintain cellular membrane during freezing and thawing (Agarwal, 2011). Generally, non-permeating cryoprotectants are not toxic to cell. Since the several permeating cryoprotectants are toxic to cells, equilibration time is the time that is required for the cryoprotectant permeate the frozen cells. The equilibration time is needed to investigate it optimum. Indeed, equilibration time varies according to types and concentrations. The equilibration time can be shortened for the cryoprotectants that rapidly permeate cell (Muchlisin, 2004; Tiersch, 2006; Agarwal, 2011.

For cryopreservation of sperm, the effects of cryomedium on viability and motility of sperm were evaluated. For example, the cryopreservation of sperm of the Asian se abass (*L. calcalifer*) was performed using ringer's solution as extender to compare two cryoprotectant of 5% DMSO and 10% glycerol (Table 2.5). Significant higher viability was obtained in the cryomedium containing 5% DMSO (84.1±1.2%) when compared with 10% glycerol (60.9±1.5%) (Palmer et al., 1993). In red sea bass (*Lutjanus argentimaculates*), cryopreservation of sperm was carried out, and the motility of sperm were evaluated. The ringer's solution was extender to compare ten types of cryoprotectants including DMSO, glycerol, PG, EG, formamide, methanol, ethanol, sucrose, trehalose, DMA (dimethyl acetamide). The result showed that the cryomedium containing ringer's solution and 5% methanal showed highest motility, suggesting to be the suitable cryomedium for red sea bass (Vuthiphandchai et al., 2009). In addition, the effects of extender and cryoprotectant on motility rate and fertilization rate frozen sperm of the European sea bass (*Dicentrarchus labrax*) were investigated. Mounib medium contain 10 % DMSO showed 75±15% of sperm motility while that of the control fresh sperm were 67±18%. The fertilization rate of fresh sperm and frozen semen were 74±15% and 70±12%, respectively (Zilli et al., 2003). Antioxidants are substances that may protect cells from the damage caused by unstable molecules known as free radicals. (Ann and Den., 1997). Supplementation of antioxidant in cryomedium was also investigated for cryoprotectant of sperm. In the European sea bass, comparing to fresh sperm, frozen sperm had significant lower motility and viability rates. However, supplementation of antioxidant such as ascorbic acid and **α**-tocopherol in cryomedium containing NAM and 10% DMSO increased

motility rate but not viability rate (Table 2.6) (Martínez-Páramo et al., 2012). Furthermore, supplementation of taurine in cryomedium containing NAM and 10% DMSO increased motility rate but not viability rate (Table 2.6) (Martínez-Páramo et al., 2013).

Table 2.3 Chemical components (g/L) and Osmolality values of extender solution used to dilute the semen.

Chemical components	**extender**				
	NAM (mM)	**NAM with ascorbic acid**	**NAM with tocopherol**	**Moubin's (mg/ml)**	**Ringer solution (g/L)**
NaCl	59.83	59.83	59.83	-	7.5
$MgCl_2$	12.91	12.91	12.91	-	-
KCL	1.47	1.47	1.47	-	0.2
$CaCl_2$	3.51	3.51	3.51	-	0.2
$NaHCO_3$	20.00	20.00	20.00	-	0.2
Glucose	0.44	0.44	0.44	-	5
ascorbic acid	-	0.1	-		-
α-tocopherol	-	-	0.1		-
BSA	1 %	1 %	1 %	10	-
Sucrose	-	-	-	42.78	-
$KHCO_3$	-	-	-	10.01	-
Reduced glutathione	-	-	-	1.99	-
pH	7.7	7.7	7.7	7.8	7.9
Osmolality	200	200	200	310	315

Table 2.4 Chemical components (g/L) and Osmolality values of extender solution used to cryopreservation of testis and ovary.

Chemical components	Extender		
	Rainbow trout		Siberian sturgeon
	Testis	Ovaries	Whole Testis/ovaries
HEPES	55.27	55.27	-
NaCl	375.48	375.48	-
KCl	7.28	7.28	-
KH2PO4	23.10	23.10	-
Na2HPO4	3.82	3.82	-
Sodium Pyruvate	3.64	3.64	-
CaCl2•2H2O	2.6	2.6	-
MgCl2•6H2O	1.4	1.4	-
Bovine serum albumin	-	-	6.67
Glucose	-	-	12
pH	7.8	7.8	-
Osmolality	270	200	290
Reference	Lee et al. (2013)	Lee et al. (2016)	Psenicka et al. (2016)

Table 2.5 Effects of extender and cryoprotectants on sperm motility and viability

Spices	Extender	Cryoprotectant	Motility (%)	Fertilization (%)	viability (%)	Reference
The Asian sea bass	Ringer's solution		-	-	80.7±1.3[a]	Palmer et al.
(*L. calcalifer*)	Ringer's solution	5% DMSO	-	-	84.1±1.20[a]	(1993)
		10% Glycerol	-	-	60.9±1.05[b]	
Red sea bass	Ringer's solution	5% DMSO	75.5±2.2[a]	-	-	Vuthiphandcha et al.
(*L. argentimaculatus*)		10% DMSO	73.3±3.9[a]	-	-	(2009)
		15% DMSO	73.3±3.9[a]	-	-	
		20% DMSO	24.4±2.2[c]	-	-	
		5% Glycerol	35.56±2.2[bc]	-	-	
		10% Glycerol	42.2±2.2[b]	-	-	
		15% Glycerol	35.5±2.2[bc]	-	-	
		20% Glycerol	24.4±2.2[c]	-	-	
		5% PG	62.2±2.2[ab]	-	-	
		10% PG	60.0±3.8[ab]	-	-	
		15% PG	24.4±2.2[c]	-	-	

Table 2.5 Effects of extender and cryoprotectants on sperm motility and viability (Cont.).

Spices	Extender	Cryoprotectant	Motility (%)	Fertilization (%)	viability (%)	Reference
Red sea bass	Ringer's solution	20% PG	17.8±2.2^{c}	-	-	Vuthiphandcha et al.
(*L. argentimaculatus*)		5% EG	68.8±2.2^{a}	-	-	(2009)
		10% EG	64.4±2.2^{a}	-	-	
		15% EG	44.4±2.2^{b}	-	-	
		20% EG	53.3±6.7^{b}	-	-	
		5% Formamide	42.2±2.2^{b}	-	-	
		10% Formamide	33.3±3.9^{bc}	-	-	
		15% Formamide	15.5±2.2^{c}	-	-	
		20% Formamide	17.8±2.2^{c}	-	-	
		5% Methanol	77.8±2.2^{a}		-	
		10% Methanol	51.1±2.2^{b}	-	-	
		15% Methanol	48.9±4.4^{b}	-	-	
		20% Methanol	40.0±3.8^{bc}	-	-	
		5% Ethanol	64.4±2.2^{a}	-	-	

Table 2.5 Effects of extender and cryoprotectants on sperm motility and viability (Cont.)

Spices	Extender	Cryoprotectant	Motility (%)	Fertilization (%)	viability (%)	Reference
		10% Ethanol	51.1±2.2^{ab}	-	-	
		15% Ethanol	42.2±2.2^{b}	-	-	
		20% Ethanol	35.5±2.2^{bc}	-	-	
		5% Sucrose	62.2±2.2^{ab}	-	-	
		10% Sucrose	53.3±6.7^{b}	-	-	
		15% Sucrose	62.2±2.2^{ab}	-	-	
		20% Sucrose	44.4±2.2^{b}	-	-	
		5% Trehalose	62.2±2.2^{ab}	-	-	
		10%Trehalose	51.1±5.9^{b}	-	-	
		15% Trehalose	51.1±5.9^{b}	-	-	
		20% Trehalose	44.4±2.2^{b}	-	-	
		5% DMA	42.2±4.4^{b}	-	-	
		10% DMA	44.4±2.2^{b}	-	-	
		15% DMA	35.5±4.5^{bc}	-	-	
		20% DMA	31.1±2.2^{bc}	-	-	
European sea bass (*D. labrax*)	Mounib medium	10% DMSO	67±18 %.	74±15%	-	Zilli et al. (2003)
	Fresh	75±15 %	70±12 %	-		

Table 2.6 Effects of antioxidant supplementations on sperm motility and viability.

Spices	extender	cryoprotectant	Motility (%)	Viability (%)	Reference
European sea bass (*D. labrax*)	NAM	10% DMSO	21.8 ±3.2	45.0	Martínez-Páramo et al. (2013)
	NAM supplemented with 1 mM taurine		30.1±3.2	45.0	
	NAM supplemented with 1 mM hypotaurine		26.7±3.5	50.0	
	Control fresh sperm		49.4±2.1	70.0	
European sea bass (*D. labrax*)	NAM	10% DMSO	20.7±3.3	42.4±5.4	Martínez-Páramo et al. (2012)
	NAM supplemented with 0.1 mM ascorbic acid		31.2±3.0	40.6±5.6	
	NAM supplemented with 0.1mM α-tocopherol		30.6±3.9	48.3±5.3	

To develop cryopreservation of testis, the effects of cryomedium on viability rate of testicular cells were evaluated. For example, the cryopreservation of testis in rainbow trout (*Oncorhynchus mykiss*) was investigated using the rainbow trout (RT) extender to compare four types of cryoprotectants including PG, EG, DMSO and GLY. The cryomedium containing RT extender and 1.3 M DMSO showed highest viability rate of spermatogonia (Lee et al., 2013). Moreover, cryopreservation of testis in Manchurian trout (*Brachymystax lenok*) was performed. The RT extender was used to compare five cryoprotectant including methanol, EG, PG, Me2SO and GLY at 1.3 M concentration. The viability of frozen testis with cryomedium contain 1.3 M methanol resulted in the highest viable cells (Lee and Yoshizaki., 2016). In tench (*Tinca tinca*), cryopreservation of testis was investigated using extender including PBS with BSA and

glucose. Six types of cryoprotectants included methanol, DMSO, DMSO+propanediol, GLY, EG and DMA were tested. There were no significant differences in the combination of extender and each cryoprotectant on the viability of spermatogonia (Linahartova et al., 2014). The cryopreservation of testis of Siberian sturgeon (*Acipenser baerii*) was performed using Siberian sturgeon extender containing 1.5 M cryoprotectant of GLY, Me2SO, EG, or mixture of Me2SO and Propanediol. The results showed that cryomedium with EG resulted in the significantly highest viability rate (17.3%±3.8%) (Psenicka et al., 2016). The cryopreservation of testis of zebrafish (*Danio rerio*) was investigated using RT extender with cryoprotectant including DMSO, EG, PG or GLY. The results showed that cryomedium contain 1.3 M DMSO had significantly highest viability rate (Marinovic et al., 2019). In Murray river rainbowfish (*melanotaenia fluviatilist*), cryopreservation of testis was carried out, and the viability of spermatogonia were evaluated. Salt solution (~296 mOsm, pH7.8) was used as extender to compare four cryoprotectant including DMSO, EG, PG or GLY, and the cryomedium containing salt solution with DMSO showed highest viability of spermatogonia (Rivers et al., 2020).

To develop cryopreservation of ovary, the effects of cryomedium on viability of ovarian germ cells was studied. For example, cryopreservation of ovary in rainbow trout (*O. mykiss*) was performed. The RT extender was used to compare four cryoprotectant including DMSO, PG, EG or GLY at 1.3 M concentration. The viability of frozen ovary with cryomedium containing 1.3 M DMSO resulted in the highest viable oogonial cells (Lee et al., 2016). In zebrafish (*D. rerio*) cryopreservation of ovary was investigated using two types of extender including L-15 solution and KCl solution containing 4 M methanol. Significant higher viability of ovarian germ cell was obtained in the KCL containing 4 M methanol (69.9±3.9%) when compared with L-15 containing 4 M methanol (4.1±0.8%) (Guan et al., 2008). The cryopreservation of ovary of Siberian sturgeon (*A. baerii*) was performed using Siberian sturgeon extender containing 1.5 M cryoprotectant of GLY, Me2SO, EG, or mixture of Me2SO and Propanediol. The results showed that cryomedium containing Me2SO, mixture of Me2SO and Propanediol or EG had significantly higher viability rate of ovarian germ cell comparing with that containing GLY (Psenicka et al., 2016)

2.2.3 Freezing methods

The freezing processes for decreasing temperature before plunging into liquid nitrogen (LN_2) is also an important factor affecting viability of frozen cells and tissues. The cooling rate is important since it affects the rate of ice crystal formation and size and solute concentration (Agarwal, 2011). During the freezing process, ice forms in and out the frozen cell which depends on cooling rate (rapid cooling and slow cooling). Rapid cooling minimizes the solution imbalance between internal and external of cell. In contrast, slow freezing leads water passing out of cell membrane which affect dehydration and shrinkage of frozen cells (Elliot et al., 2017). For cryopreservation of sperm of the red sea bass (*L. argentimaculates*), the effects of cryomedium and cooling rate were investigated. The cryomedium containing ringer's solution and each DMSO, PG, EG, methanol or ethanol at different concentrations (5 % or 10%) was examined with the different cooling rate. The cooling rate at -3°C/min, -5°C/min, -10°C/min or -15°C/min with different 2 final temperatures (-40°C and -80°C) before plunged into LN_2 were studied. The results showed that cryomedium containing 10% DMSO at final temperature -80°C (-10°C/min) showed significant highest motility rate (91.1±2.2%) and viability rate (92.7±2.3%) (Vuthiphandchai et al., 2009) (Table 2.7). Moreover, cooling rate affects frozen tissue because of the complexity of tissue morphology. The freezing rate (time of decreasing temperature) should be optimized for particular species (Picton et al., 2000). For example, a uniform cooling rate of 1°C per minute from ambient temperature was reported to be effective for a wide variety of cells and organisms (Simione, 2012). For example, the cooling rate of 1°C per minute was used for cryopreservation of testis and/or ovary in rainbow trout (Lee et al., 2013), zebrafish (Marinovic et al., 2019), Siberian sturgeon (Psenicka et al., 2016), Murray river rainbowfish (Rivers et al., 2020) and tench (Linahartova et al., 2014). Cryopreservation of testis in Manchurian trout (*B. lenok*) was performed. The cryomedium containing RT extender and methanol at 1.3 M was used to compare four different cooling rates including -0.5°C/min, -1°C/min, -10°C/min and -20°C/min using a computer-controlled rate freezer. The result showed that the highest survival rate was obtained when using a cooling rate at -1°C/min (Lee and Yoshizaki., 2016).

2.2.4 Thawing process

In generally, thawing process is also important factor affecting the viability of frozen cell (Mazur et al., 1970; chua and chou, 2009). Indeed, the thawing process is investigated to minimize the damage associated with recrystallization (the fuse of small ice crystal into large crystals during thawing). Indeed, intracellular ice formation is thought to be main cause of cryoinjury (Tiersch, 2006). Therefore, the thawing condition after cryopreservation have effects on cell viability. Therefore, investigation of optimum thawing process is necessary to develop suitable cryopreservation of cell/tissue. There have been several studies demonstrating the optimum of thawing process of frozen sperm. For instant, the thawing condition of 35°C for 15 sec, 30°C for 2-3 min and 70°C 5 sec was used to thaw the frozen sperm of the European sea bass, the Asian sea bass and red sea bass, respectively (Zilli et al., 2003; Palmer et al., 1993; Vuthiphandchai et al., 2009). For cryopreservation of testis and ovary, the thawing condition was also examined. For example, for frozen testis and ovary in rainbow trout, the thawing condition was 10°C (Lee et al., 2013). The thawing condition of 38°C for 40 sec was used frozen testis in tench (Linahartova et al., 2014). In addition, the thawing condition of 25°C for 2 min was used for frozen testis in zebrafish (Marinovic et al., 2019). Moreover, the thawing condition of 30°C for 1 min was applied for frozen testis in Murray river rainbowfish (Rivers et al., 2020). For cryopreservation of testis of Manchurian trout (*B. lenok*), cryomedium containing RT extender and 1.3 M methanol and cooling rate at -1°C/min was used to compare four different thawing rates including 10°C, 20°C, 30°C or 40°C for 1 min. The result showed that the highest viability of spermatogonia was obtained with the thawing rate at 30°C for 1 min (81.0±1.3%) (Lee and Yoshizaki., 2016).

Table 2.7 Percentage motility and viability in cryopreservation at different temperature in freezing process of sperm.

spices	extender	cryoprotectant	Final temperature and time	Motility (%)	Viability (%)	Reference
Red sea bass	Ringer's	5% DMSO	-40°C (3°C/min)	11.1±2.2[b]	30.1±2.7[a]	Vuthiphandcha et al.
(*L. argentimaculatus)*	solution	10% DMSO		17.8±2.2[a]	29.1±4.6[a]	(2009)
		5% EG		2.2±2.2[c]	24.8±2.8[a]	
		10% EG		2.2±2.2[c]	21.6±1.8[a]	
		5% PG		0[c]	25.1±3.9[a]	
		10% PG		8.8±2.2[bc]	26.6±5.4[a]	
		5% Ethanol		0[c]	25.3±2.1[a]	
		5% methanol		4.4±2.2[c]	20.7±0.9[a]	
		5% DMSO	-40°C (5°C/min)	48.9±8.8[b]	49.6±5.5[b]	
		10% DMSO		68.9±4.4[a]	73.2±5.6[a]	
		5% EG		48.9±4.4[b]	52.5±4.9[b]	
		10% EG		15.5±2.2[d]	27.5±1.9[c]	
		5% PG		8.8±2.2[de]	26±2.8[c]	
		10% PG		11.1±2.2[d]	29±6.1[c]	
		5% Ethanol		22.2±2.2[c]	27.9±5.4[c]	
		5% methanol		11.1±2.2[d]	17.4±1.4[c]	

Table 2.7 Percentage motility and viability in cryopreservation at different temperature in freezing process of sperm (Cont).

spices	extender	cryoprotectant	Final temperature and time	Motility (%)	Viability (%)	Reference
Red sea bass (*L. argentimaculatus)*	Ringer's solution	5% DMSO	-40°C (10°C/min)	8.8±2.2^{bc}	51.6±6.5^{a}	Vuthiphandcha et al. (2009)
		10% DMSO		2.2±2.2^{c}	47.6±1.7^{a}	
		5% EG		28.8±2.2^{a}	33.3±2.8^{ab}	
		10% EG		15.5±2.2^{b}	31.8±5.5^{ab}	
		5% PG		11.1±2.2^{b}	34.9±2.2^{ab}	
		10% PG		8.8±2.2^{bc}	35.9±4.9^{ab}	
		5% Ethanol		2.2±2.2^{c}	29.2±3.5^{b}	
		5% methanol		4.4±2.2^{c}	35.6±5.1^{ab}	
		5% DMSO	-40°C (12°C/min)	4.4±2.2^{ab}	48.8±7.4^{a}	
		10% DMSO		11.1±2.2^{a}	62.7±6.7^{a}	
		5% EG		11.1±2.2^{a}	32.3±2.6^{b}	
		10% EG		8.8±2.2^{a}	30.9±3.7^{b}	
		5% PG		4.4±2.2^{ab}	29±5.4^{b}	
		10% PG		8.8±2.2^{a}	18.6±1.3^{b}	
		5% Ethanol		0^{b}	26.1±6.9^{b}	
		5% methanol		4.4±2.2^{ab}	17.7±3.1^{b}	

Table 2.7 Percentage motility and viability in cryopreservation at different temperature in freezing process of sperm (Cont.)

spices	extender	cryoprotectant	Final temperature and time	Motility (%)	Viability (%)	Reference
		5% DMSO	-80°C (3°C/min)	11.1±2.2^{s}	37.5±0.7^{a}	
		10% DMSO		15.5±2.2^{a}	43.4±4.6^{a}	
		5% EG		0^{bc}	24.4±1.0^{ab}	
		10% EG		0^{bc}	26.1±3.8^{ab}	
		5% PG		0^{bc}	27.6±3.8^{ab}	
		10% PG		8.8±2.2^{ab}	17.0±4.5^{b}	
		5% Ethanol		0^{bc}	31.9±4.2^{a}	
		5% methanol		4.4±4.4^{b}	25.9±2.8^{b}	
		5% DMSO	-80°C (5°C/min)	4.4±2.2^{a}	68.4±2.7^{a}	
		10% DMSO		4.4±2.2^{a}	72.1±2.3^{a}	
		5% EG		0^{ab}	59.3±4.1^{a}	
		10% EG		0^{ab}	37.6±1.3^{b}	
		5% PG		0^{ab}	40.4±1.4^{b}	
		10% PG		0^{ab}	41.9±5.3^{b}	
		5% Ethanol		6.6±0^{a}	35.5±2.4^{b}	
		5% methanol		8.8±2.2^{a}	42.6±0.6^{b}	

Table 2.7 Percentage motility and viability in cryopreservation at different temperature in freezing process of sperm (Cont.)

spices	extender	cryoprotectant	Final temperature and time	Motility (%)	Viability (%)	Reference
		5% DMSO	-80°C (10°C/min)	71.1±2.2^{b}	74.7±3.1^{b}	
		10% DMSO		91.1±2.2^{a}	92.7±2.3^{a}	
		5% EG		11.1±2.2^{d}	44.1±1.7^{c}	
		10% EG		6.6±0^{e}	39.5±4.9^{c}	
		5% PG		0^{f}	63.4±8.1^{b}	
		10% PG		28.8±2.2^{c}	36.5±4.9^{c}	
		5% Ethanol		0^{f}	31.7±1.1^{C}	
		5% methanol		6.6±0^{e}	33.2±0.8^{c}	
		5% DMSO	-80°C (12°C/min)	31.1±2.2^{a}	69.0±6.3^{a}	
		10% DMSO		17.8±2.2^{b}	77.6±3.2^{a}	
Red sea bass	Ringer's	5% EG	-80°C (12°C/min)	6.6±0^{c}	55.2±4.0^{a}	Vuthiphandcha et al.
(*L. argentimaculatus)*	solution	10% EG		0^{cd}	61.8±2.8^{a}	(2009)
		5% PG		0^{cd}	51.6±5.2^{b}	
		10% PG		6.6±0^{c}	45.3±6.2^{b}	
		5% Ethanol		0^{cd}	49.6±7.4^{b}	
		5% methanol		4.4±1.8^{c}	40.2±8.4^{b}	

2.3 Germ cell transplantation

Germ cell transplantation is a technique for injection of germ cell from doner fish into the gonad of recipient fish. Generally, germ cell that exhibit translatability including primordial germ cell (PGCs), spermatogonia and oogonia. In germ cell transplantation viable between doner-recipient relationships including intra-genus, inter-genus and inter family. transplanted germ cell could incorporate, colonize and proliferate in recipient gonad, and the recipient fish is grown through maturation to become broodstock. These broodstock produce donor-offspring, and the broodstock is therefore called surrogate broodstock. Germ cell transplantation is the only functional approach for investigation of spermatogonia stem cell biology (Brinster and Avarbock, 1994; Brinster and Zimmermann, 1994). The newly hatched larvae do not have a mature immune and thus is able to use as recipient larvae (Takeuchi et al., 2004; Okutsu et al., 2007). Generally, during early gonadal development, the primordial germ cell (PGCs) which is a progenitor cell of germ cell migrate towards genital ridges (future side of gonad) by chemotaxis using pseudopodia (Yoshizaki et al., 2002). The PGCs incorporate in genital ridge, develop to become gonad and differentiate to become oogonia in female and spermatogonia in male. There are three major applications of germ cell transplantation technology. One objective of germ cell transplantation was to study fundamental aspects of male germ line stem cell biology and male fertility (Dobrinski, 2008).

In fish, germ cell transplantation was conducted by isolation of undifferentiated germ cell, labelling cells with fluorescent such as PKH26 and antibody and then transplanted under stereomicroscope into the peritoneal cavities in recipient fish. The transplanted germ cell can be differentiated with recipient fish which depend on the sex of the recipient fish (Takeuchi et al., 2003; Okutsu et al., 2006). In salmonids, Okutsu et al. (2006) develop germ cell transplantation using spermatogonia as donor cells. The undifferentiated spermatogonia could colonize and sexually differentiated in recipient gonad. In addition, the transplanted fish produced functional sperms and eggs. Moreover, the transplanted fish produced donor-derived offspring (Takeuchi et al., 2004; Okutsu et al., 2007). Farlora et al. (2013) studies intraperitoneal germ cell transplantation in Nile Tilapia. Transgenic Nile Tilapia carrying green fluorescent protein gene was used doner fish for isolation spermatogonia for germ cell

transplantation. The transplanted testicular testis was dissociated using the enzyme solution (0.5% trypsin,5% FBS, 0.05% DNase I). The isolated testicular cell was stained with PKH26 fluorescent. For transplantation, isolated testicular cells (20,000 cells) were microinjected into peritoneal cavity of newly hatched Nile Tilapia. Subsequently, after 22 days post-transplantation, the genital rides of the recipient fish were observed to check colonization. The colonization of the transplanted cells in gonad of recipient fish were demonstrated (Farlora et al., 2013). In addition, germ cell transplantation was developed in sturgeon. The Siberian sturgeon was used as donor fish for isolation spermatogonia or oogonia to transplant into sterlet larvae fish. After transplantation, the survival rate was not different between control and transplantation (96.7% and 95.8%, respectively). The transplanted cells were observed in the genital ridge of recipient fish at 30 days post transplantation (dpt) (Martin et al., 2015).

Germ cell transplantation was also conducted using cryopreserved cell as donor fish. Kobayashi et al. (2007) conducted cryopreservation of primordial germ cell and subsequently transplantation in salmonid fish. Transgenic rainbow trout (*Oncorhynchus mykiss)* carrying green fluorescent protein gene driven by *vasa* promoter (*Pvasa-GFP* transgenic strain) was used as doner fish for isolation of PGC for cryopreservation. In the cryopreservation method, PBS-based medium contain 1.8 M ethylene glycol was used as extender. For transplantation, the frozen PGCs were microinjected into the peritoneal cavities of non-transgenic fish. After 30 days post-transplantation, the colonization of the transplanted cells was observed under a fluorescent microscope. The PGCs with GFP-positive cells were observed in the genital ridge of recipient fish at 30 days post transplantation(dpt). The colonization rate of control fresh PGC was 12.5±4.8% while that of the 1-day frozen PGC and 10-month frozen PGC were 10.1±3.9% and 20.6±11.9%, respectively. Moreover, the transplant recipient male and female produced doner-derived offspring (Kobayashi et al., 2007). Cryopreservation of whole testis was used for transplantation in salmonids. The rainbow trout (*O. mykiss) Pvasa-GFP* transgenic strain was used as doner fish, and cryopreservation of whole testis was performed. The wild type of triploid rainbow trout was used as recipient fish. Determination of viability rate was performed using flow cytometry and trypan blue (TB) staining. The extender used was 35.2% extender

containing 1.3 M DMSO at freezing rate -1°C and thawing at 10°C for 5 min showing high viability of spermatogonia. The frozen testicular were dissociation, and approximately 5,000 spermatogonia cells were transplanted into wild type triploid rainbow trout. After 20 days post-transplantation (dpt), the transplanted spermatogonia were incorporated into genital ridge of recipient fish. After 31 dpf, the transplanted germ cells were observed to proliferate within genital ridge in recipient fish. After 2 years, male triploid fish could produce doner-derived of sperm. Also, after 3 years, female triploid fish could produce doner-derived egg. The transplant recipient fish were able to produce donor-derived offspring (Lee et al. 2013). Moreover, cryopreservation of testis from Manchurian trout (B. *lenok*) was performed and transplanted into wild type triploid rainbow trout. The colonization rate was 89.0 ±5.5% for control fresh spermatogonia and 84.1±7.4% for frozen spermatogonia. After 45 days post-transplantation, spermatogonia cell were rapidly proliferated in gonad (Lee and Yoshizaki, 2016).

CHAPTER III

MATERIALS AND METHODS

3.1 Broodstock management

Immature male of the Asian sea bass (300–500 g) were reared within a cage (2 × 24m) in the earthen pond at the SUT farm, Suranaree University of Technology, Nakhon Ratchasima, Thailand. Fish were fed daily, twice a day, at a rate of 3% of the total body weight per day with commercial pellets.

3.2 Research station

SUT Farm (fisheries station) and equipment building F10 in Suranaree University of Technology.

3.3 Venue of the Study

Suranaree University of Technology Farm (Aquaculture), Instrument Building 10 and 14, Science and Technology Equipment Center Suranaree University of Technology.

1) Fish

All experiments were followed ethical principles and guidelines for the use of Animals for Scientific Purposes (ACT) for care and use of animal in the laboratory of fish at Suranaree University of Technology (SUT) Nakhon Ratchasima, Thailand. Immature Asian sea bass aged 4-5 mouths were collected from fish farm in Nakhon Sawan, and recipient fish were bought from fish larvae farms in Chonburi or Chachoengsao, Thailand and kept at the SUT farm, Suranaree University of Technology until testes cryopreservation and germ cell transplantation.

2) Histopathology

The gonadal development of testes collected from different size; 75 g, 100 g, 300 g, 500 g, 750 g, 1.0 kg, 3.0 kg, 8.0 kg, and 11.0 kg were sampled for histological study. The testes were immediately fixed with bouin's solution for 16 to

24 hr. and maintained in the 80% (v/v) ethanol at 4°C. then, dehydrated at room temperature in graded ethanol of 90% for 60 min once and 100% for 20 min three times. The gonadal tissues were immersed in the ethanol:buthanol at the ratio of 4:1, 3:2, 2:3, and 1:4 for 30 min in each concentration. After that, the samples were soaked in the 100% buthanol, twice for 20 min, and three times in 100% xylene for 20 min prior to embedded in the paraffin. The embedded gonadal tissues were section at 5 µm of thickness in a rotary microtome (HMT-2258) and stained with hematoxylin and eosin (H & E). Images of sections were obtained using a compound microscope (Olympus BX53F2, Shinjuku-ku, Tokyo, Japan)

Table 3.1 Embedding process.

Solution	Soaking time
90 %ethanol	1 hour or overnight
First absolute ethanol	20 minutes
Second absolute ethanol	20 minutes
Third absolute ethanol	20 minutes
Ethanol and butanol ratio by 4:1	30 minutes
Ethanol and butanol ratio by 3:2	30 minutes
Ethanol and butanol ratio by 2:3	30 minutes
Ethanol and butanol ratio by 1:4	30 minutes
First 100 %butanol	30 minutes
Second 100 %butanol	30 minutes
Butanol and xylene ratio by 1:1	20 minutes
First 100 %xylene	20 minutes
Second 100 %xylene	20 minutes
Xylene and paraffin ratio by 1:1	20 minutes
Solution	**Soaking time**
First paraffin	30 minutes
Second paraffin	30 minutes

3) Testes preparation

Fish were cleaned, and body weight, and total length were recorded. Indeed, all Immature fish were shortly anesthetized by used 10% (v/v) clove oil before collection the gonad (testis) through peritoneal dissection technique. First, scissors were used to make an incision along the midline of the body cavity, and the whole

testes were removed. Testes weight 0.07±0.05 g and gonadosomatic index; GSI (gonad weight/body weight x 100) 0.02±0.01. Second, the testes were placed in 24 well plate of Leibovitz's L-15 Medium (pH 7.8, Gibco Invitrogen Co., Grand Island NY, USA) and a combination with 100 unit/ml penicillin (pH 7.1; 17-602E, Lonza Group AG, MD, USA). Next step, the testes were removed connective tissue, fat, blood clots, and another three washes with L-15 medium. finally. the testes were weighed using analytical balance for cryopreservation of testes using slow-freezing method.

3.4 Cryopreservation of testes

3.4.1 Effect of extender and cryoprotectant

The whole testes were transfer into 1.8 mL cryovial (43012, SPL Life Sciences) containing 500 µL cryomedium including Moubin's medium, Non-Activating Medium (NAM), or Leibovitz's L-15 medium that comprised cryoprotectants including dimethyl sulfoxide (DMSO), ethylene glycol (EG), or propylene glycol (PG). In total, there were nine combinations (3 extender x 3 cryoprotectant) and the fresh control. The samples were equilibrated on ice for 60 min. Then, cryovial were cooled at a rate of −1°C/min for a period of 90 min by using bicell plastic freezing container in a deep freezer (−80°C) before immediately transfer into liquid nitrogen (LN_2).

3.4.2 Effect of concentration

The testes samples were distributed in 1.8 mL cryotube containing 500 µL of cryomedium L-15 medium supplement with DMSO at concentrations of 7.5%, 10%, 12.5% or 15%. therefore, there were 4 treatments. then, all sample were equilibrated and frozen, as above.

3.4.3 Effect of thawing condition

Different thawing condition were tested using thawing temperature (10°C or 28°C) and thawing times (4 minute, 8 minute or 10 minute) based on section 4.1 and 4.2. In this study, six treatment combinations (2 temperatures x 3 times) and the fresh control. Thawed testes were rehydrated in three changes of L-15 medium and holed in 24 well plate with L-15 medium for dissociation.

Table 3.1 Combination treatment in experimental I.

Treatment	Factor 1 (Extender solution)	Factor 2 (cryoprotectant solution)
1	Moubin's medium	DMSO
2	Moubin's medium	EG
3	Moubin's medium	PG
4	NAM	DMSO
5	NAM	EG
6	NAM	PG
7	L-15	DMSO
8	L-15	EG
9	L-15	PG

Table 3.2 Chemical composition of extender solutions.

Chemical components	Extender		
	Moubin's (mg/ml)	NAM (mM)	L-15*
NaCl	-	59.83	-
$MgCl_2$	-	12.91	-
KCL	-	1.47	-
$CaCl_2$	-	3.51	-
$NaHCO_3$	-	20.00	-
Glucose	-	0.44	-
BSA	10	1 %	-
Sucrose	42.78	-	-
$KHCO_3$	10.01	-	-
Reduced glutathione	1.99	-	-
L-15	-	-	13.7
DI water	-	-	1 L
pH	7.8	7.7	7.8
Osmolality	310	200	215
Reference	Zilli et al. (2012)	Martínez-Páramo et al. (2013)	Yoshikawa et al. (2018)

Annotation * L-15 medium contains Inorganic Salts, Amino Acids, Vitamins and other substances (Appendix 1.)

Table 3.3 Combination treatment in experimental II.

Treatment	Extender	Concentration of DMSO
1	L-15	7.5 % DMSO
2	L-15	10 % DMSO
3	L-15	12.5 % DMSO
4	L-15	15 % DMSO

Table 3.4 Combination treatment in experimental III.

Treatment	Temperature	time
1	10°C	4 min
2	10°C	8 min
3	10°C	10 min
4	28°C	4 min
5	28°C	8 min
6	28°C	10 min

3.5 Spermatogonia cell isolation

Fresh and post-thawed testes were placed onto a sterile glass plate and cut gently into small pieces using a De-Weckers iris scissors on ice. The small pieces were incubated with the dissociation enzyme containing; 0.4% Collagenase H (Roche Diagnostics, Mannheim, Germany), 0.03% Dispase II (Sanko Junyaku Co., Ltd., Tokyo, Japan) 10% Fetal bovine serum (FBS, Gibco Invitrogen Co., Ltd., Grand Island NY, USA), 900 U/mL DNase I (Roche Diagnostics, Mannheim, Germany), diluted in the L-15 (pH 7.8, Gibco Invitrogen Co., Grand Island NY, USA), at a ratio of 0.2 g of gonadal tissue to 1 mL of dissociation enzyme. The samples were gently mixed by pipetting every 30 min for 2 hr. of incubation periods at 37°C. After the incubation with dissociation enzyme, the cell suspension was filtrated through cell strainer (40 µm nylon screen; Life Sciences, One Becton Circle, Durham, USA) to elimination dissociated cell clumps. then, three washes with L-15 medium. The dissociated cell suspension in L-15 medium and maintained at 4°C until observation and using in the experiment.

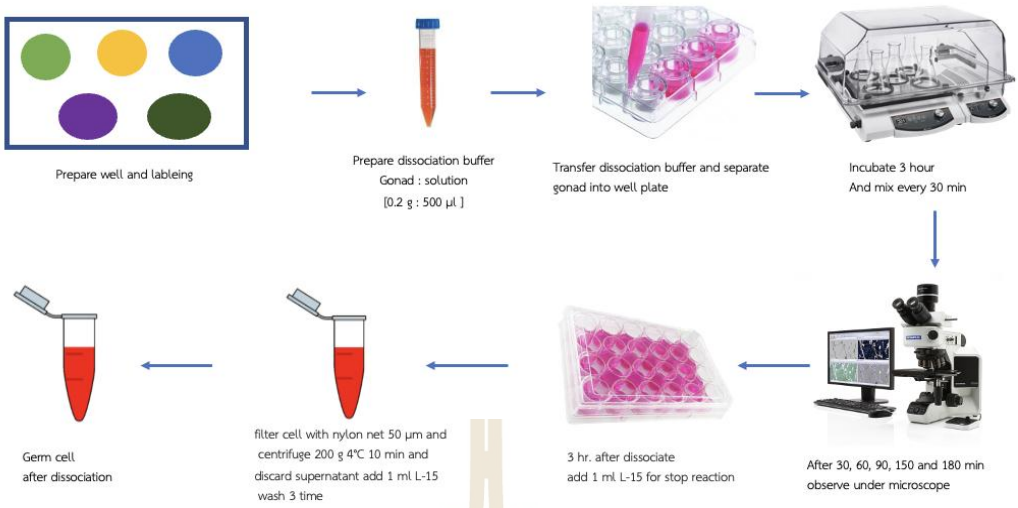

Figure 3.1 Dissociation method for gonad of Asian sea bass fish.

3.6 Assessment of Cell Viability

3.6.1 Trypan blue staining method

The spermatogonial cell were stained with 0.4% trypan blue at the ratio of 1:1 (v/v) to differentiate live and dead cell and incubated for 5 minute at room temperature. Then, the sample was pipetted onto a hemacytometer. Cells were observed under an Olympus microscope.

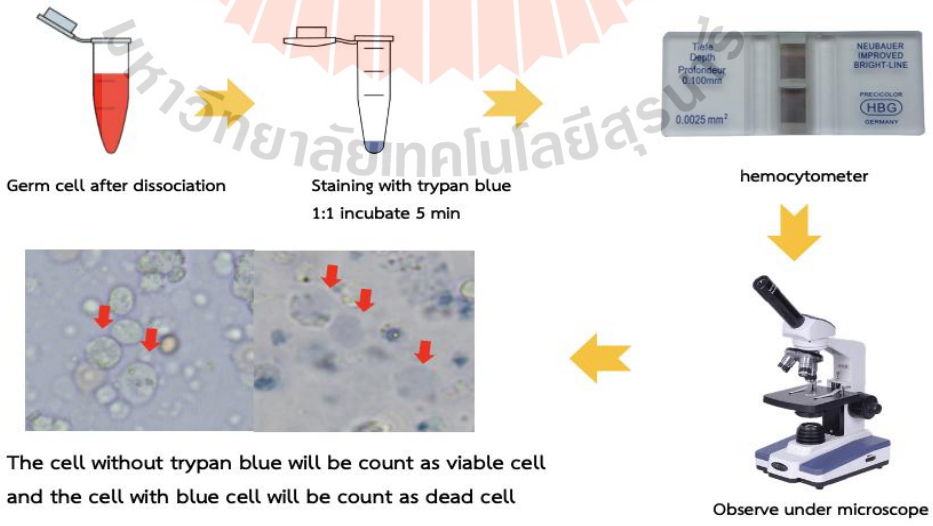

Figure 3.2 Dissociation method for gonad of Asian sea bass fish Indeed, dead cells were stained blue and live cells remained unstained.

3.6.2 Fluorescein diacetate-propidium iodide staining and flow cytometric analysis

To validate the viability rate between fresh and post-thawed spermatogonial cell, fluorescein diacetate (FDA)-propidium iodide (PI) staining following by flow cytometric analysis was performed. Simultaneous double-staining of FDA and PI was performed by gently mixing 5 µl of FDA solution (5 mg/ml in acetone) and 5 µl of PI (0.02 mg/ml in PBS) in 100 µl of cell suspension at room temperature in the dark for 3 min. Viable cell was assessed by epifluorescence microscopy and by flow cytometry. Using fluorescence microscope with 520 nm and 590 nm filters, FDA produces green fluorescence in viable cells while PI produces red fluorescence in dead cells. In addition, the stained cell suspension was analyzed using a DxFLEX Flow Cytometer (Beckman Coulter, IN, USA). Red and green fluorescence signals were detected using the Argus FITC filter block. Fluorescent histograms were gated by forward light scatter to eliminate noise. A typical histogram comprised of data from 20,000 cells measured at a flow rate of 60 µl per sec.

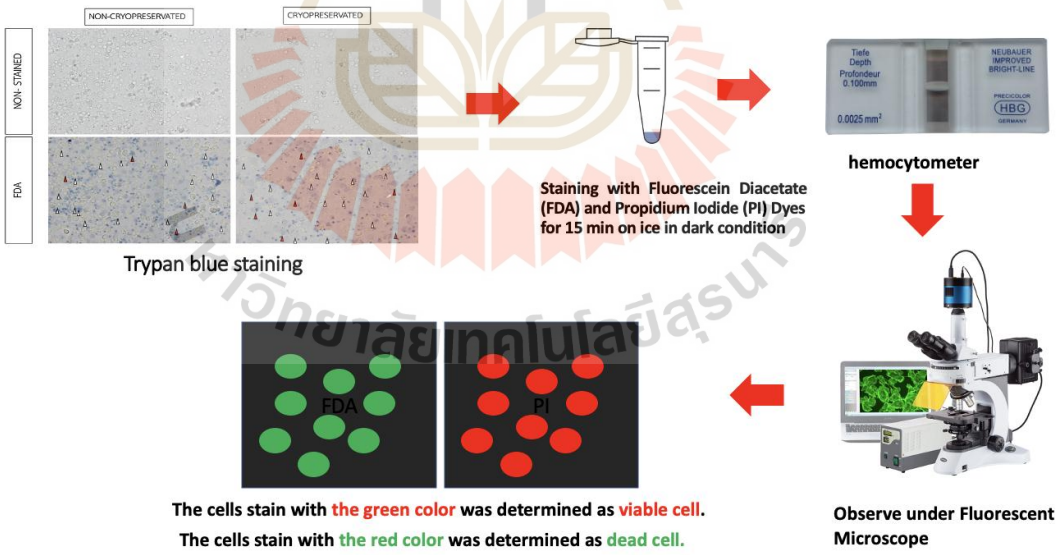

Figure 3.3 Fluorescein diacetate and propidium iodide stanning.

3.6.3 Apoptosis Quantitation

The apoptosis in spermatogonial cells was identified by an Annexin V affinity assay, using the apoptosis detection kit (Biolegend, CA, USA) and flow cytometry. Annexin V was used to quantify changes in the plasma membrane (phosphatidylserine(PS) translocation) of spermatogonial cells. Fresh and frozen testis were dissociated, washed three times in L-15 medium (GIBCO BRL), and then approximately 10^7 cells were resuspended in 100 µl of Annexin V binding buffer. Cells were then incubated with 5µl of fluorescein isothiocyanate (FITC)-conjugated Annexin V and 10µl of PI for 15 min in the dark at room temperature. Subsequently, the cells were then resuspended in 400µl of Annexin V binding buffer prior to the flow cytometric analysis. The cell suspension without staining was used as negative control. A total of 20,000 events were analyzed at flow rate of 60 µl/min for each sample. Two-color analysis of apoptosis within the mononuclear cell population was carried out using a DxFLEX Flow Cytometer (Beckman Coulter). Fluorescence compensation on the flow cytometer was adjusted to minimize overlap of FITC and PI signals. Analysis was based on gating a subpopulation of cells by forward scatter (FSC) versus side scatter (SSC). The proportion (%) of testicular cell positive for Annexin V but negative for PI (AnnexinV+/PI-) which was considered as proportion of early apoptotic-like cells divided by the total number of cells in the gated region. The AnnexinV+/PI+ testicular cells were considered as necrotic cells.

3.6.4 In situ hybridization

In situ hybridization with a vasa probe was conducted against the cell smears that were made from unstained and antisense cells. Dissociated cells were prepared from 300- 500 g body weight, and then approximately $1–2\times10^5$ cells were sorted and smeared on glass slides (Matsunami, Osaka, Japan) Next, in situ hybridization with a vasa probe was performed against the cell smears. RNA probes were synthesized from a cDNA vasa fragment of the Asian sea bass. Probe synthesis and hybridization were performed according to the protocol.

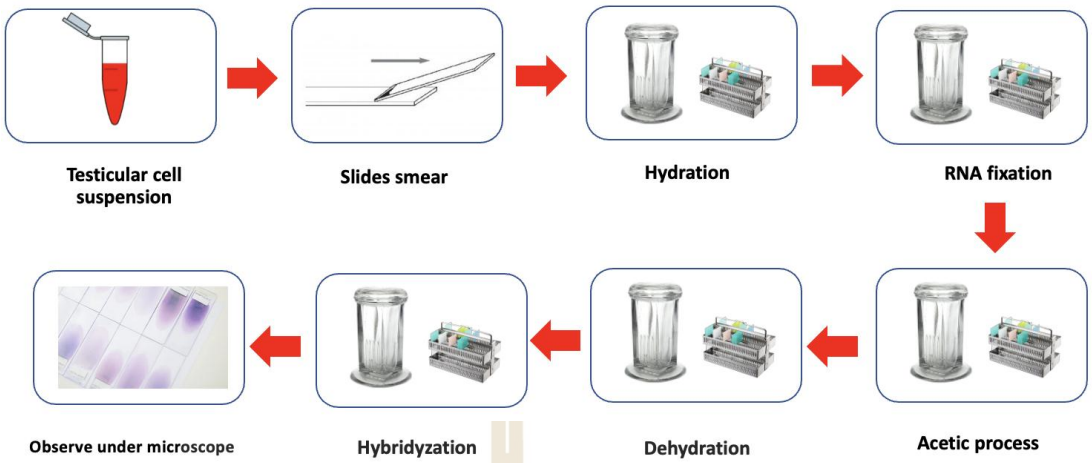

Figure 3.4 In situ hybridization method.

3.7 Transplantation assay

Before germ cell transplantation into the gonad of allogenic recipients, testicular germ cells were incubated with the fluorescents membrane dry PKH26 (PKH26 Cell Linker Kit, Sig- maeAldrich), which is easily to detect the transplanted doner cell in the genital ridge of larvae. The recipient fish were anesthetized by tricaine methanesulfonate (MS-222) in sea water (30 ppt). PKH26-labeled cells were transplanted at 10 days post hatch (dph) into peritoneal cavity of the Asian sea bass hatching larvae (total length 4.0 mm). After transplantation, recipient fish were transferred to a recovery tank with 10% Bovine Serum Albumin solution (BSA). Recipient fish were raised in 80 liter sea water tank and feeding artemia (*Artemia salina)* to the tank twice daily.

3.8 Observation of spermatogonia labeled in recipients

The percentage that transplanted spermatogonia PKH26-labled cells colonized into genital ridges of 24 day post transplantation (dpt.) total length 9.0 mm under fluorescent compound microscope (Olympus BX53F2, Leica Microsystems, Tokyo, Japan) the head and digestive organs were dissected and removed. The recipient fish was fixed in 4% Paraformaldehyde (PFA) for 5 min of ice and holed in PBS. Their gonad was observed under fluorescent compound microscope (Olympus BX53F2 and

Olympus DP74). Colonization efficiencies were calculated using the following formular: colonization rate = [(number of colonization at genital ridges of recipients)/ (number of fish observed)] x 100.

3.9 Statistical analysis

Data are presented as means ± standard deviation (SD). Statistical significance using one-way analysis of variance (ANOVA) were determined significance differences among group. Differences between two group were also analyzed by student's t test. Overall, for all statistical analysis, differences were determined statistically significant when the respective *p*-value was <0.05.

CHAPTER IV

RESULTS

4.1 Testis of Asian Sea bass

This study performed sampling of immature male to determine the size of fish in which its testes contained high proportion of spermatogonia cells. The fish have body weight (BW) of ~ 75g (GSI = 0.075%), ~ 100 g (GSI = 0.127%), ~ 300 g (GSI = 0.177%), ~ 500 g (GSI = 0.277%), ~ 750 g (GSI = 0.511%), ~ 1.0 kg (GSI = 0.721%), ~ 3.0 kg (GSI = 0.862%),~ 8.0 kg (GSI = 0.922%), and ~ 11.0 kg (GSI = 1.324%) were sampling for histological study (Figure 4.1). In general, the typical appearance of testicular cell which has diameter of ~ 10 µm with relatively large nucleus and a few compact nucleoli was identified as spermatogonia. A spermatogonium is an undifferentiated male germ cell. spermatogonia undergo spermatogenesis to form mature spermatozoa in the seminiferous tubules of the testis. The result showed that the fish at 75-500 g BW have testes containing high proportion of spermatogonia [(SG); red arrowhead] while various stage of testicular cells including primary spermatocyte [(PSC); yellow arrowhead], secondary spermatocytes [(SPC); orange arrowhead], and spermatid [(SZ); green arrowhead] were observed in testes of fish at ~ 500 g BW (Figure 4.1). Therefore, the fish at ~ 300 - ~ 500 g BW was use throughout this study. Indeed, its testes contained spermatogonia like cell ~ 3.08×10^6 cell/g testes.

Table 4.1 Number of spermatogonial cells to demonstrate suitable stage for cryopreservation.

Gonad	body weight (g)	Total cell/1 g (cell)	Cell approximately 10 µm / 1 g (cell)	Proportion of 10 µm cells/1 g (%)
Testes	75-100	2,532,000	910,000	35.94
	300-500	6,741,667	3,081,000	45.70
	750-1000	10,431,225	1,580,000	15.15

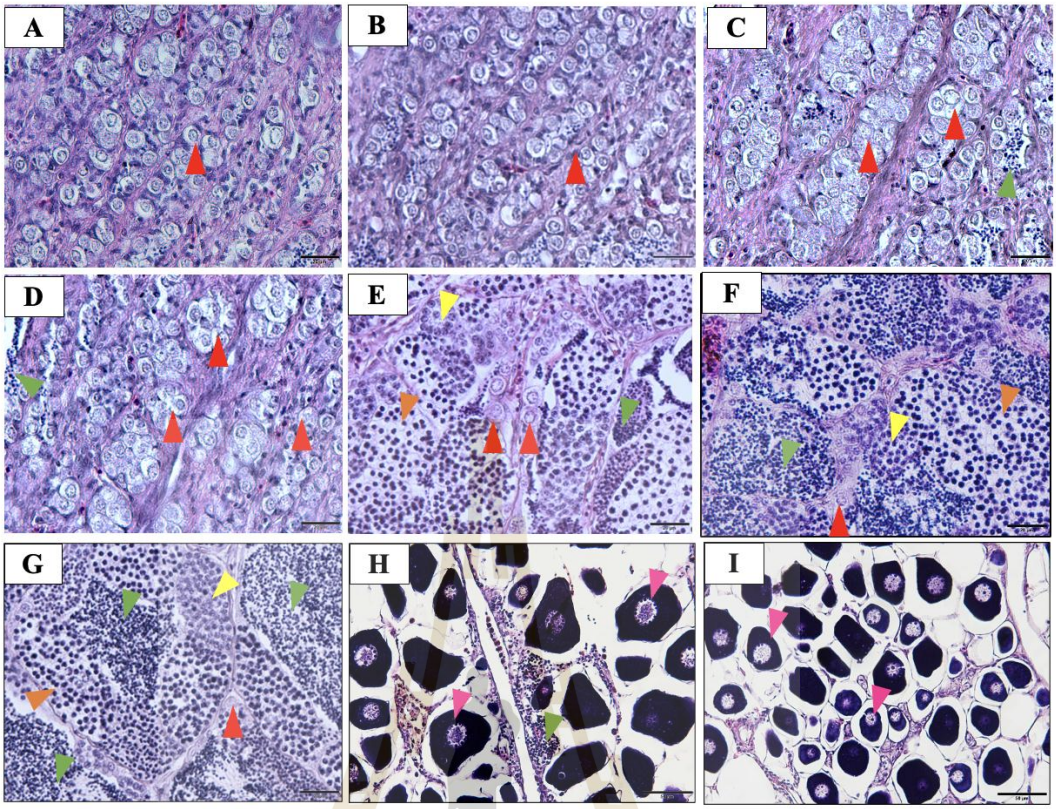

Figure 4.1 Histological study of donor testis of the Asian sea bass. Testis were collected from the Asian sea bass 75 g to 11.0 g body weight. (Scale bars = 50 μm).

4.2 Cryopreservation of testis in the Asian sea bass

For optimization of slow-freezing protocol for tissue freezing of the Asian sea bass testes, suitable cryomedium containing extender, cryoprotectants were determined. The suitable cryomedium containing various extender (Mounib, NAM or L-15) and cryoprotectants (DMSO, EG, or PG) were tested (Table 3.1). The percentage of membrane-intact cells presenting viability rate of spermatogonia of fresh and cryopreserved testes was assessed using trypan blue staining (Figure 4.10). Among these cryomedium, the viability rate of spermatogonia cryopreserved with cryomedium containing L-15 and DMSO was significant highest ($P<0.05$). In this study subsequently investigate suitable concentration (7.5%, 10%, 12.5%, or 15.0%) of DMSO cryoprotectants. Using L-15 extender, DMSO at 10 % shown yielded significantly highest viability rate ($P<0.05$) (Table 4.2). The effects of thawing condition

including temperature and time were investigated. Our result showed that the highest viability rate of spermatogonia was obtained for frozen testes with thawing process at 10°C for 8 min ($P<0.05$) (Table 4.3). Therefore, the suitable cryopreservation conditions for testes of the Asian seabass were cryomedium containing L-15 and 10% DMSO with thawing conditions at 10°C for 8 min which was used throughout this study.

In this study, using flow cytometric analysis with permeable esterase substrate fluorescein diacetate (FDA) and the cell impermeable DNA stain propidium iodide (PI), viability rate of spermatogonia from fresh and cryopreserved testes was also performed. The viability rate of spermatogonia from cryopreserved testes (81.63± 0.92%) was no significant different of fresh testes (81.06±1.11%) ($P<0.05$) (Figure 4.17 and 4.18). To test whether cryopreservation induced apoptosis, a typical two-dimensional flow cytometric profile of dissociated cells labelled with Annexin V and PI was showed in (Figure 4.19 and 4.20). The majority of the cells from fresh testes (45.06+3.34 %) and cryopreserved testes (37.57+2.28 %) which fell in the lower left quadrant, were live cells (Figure 4.19 and 4.20). Indeed, live cells from fresh testes were significantly higher than that from cryopreserved testes ($P<0.05$) (Figure 4.19 and 4.20). Moreover, comparing with fresh testes (23.48+2.38%), lower percentage of dead cell (18.66+1.66%) was observed in cryopreserved testes. Indeed, fraction of early (Annexing V+, PI-; 26.08+1.82%) and late apoptotic cells (Annexing V+, PI+; 17.19+2.62%) in cryopreserved testes was significantly higher than that obtained from fresh testes (Annexing V+, PI-; 16.38+2.33%; Annexing V+, PI+; 14.47+3.26%) ($P<0.05$) (Figure 4.19 and 4.20).

Table 4.2 Number of dissociated cells and their viability rates of frozen testes (Mean±SD; N=6).

Extender	cryoprotectant	Number of total isolate cells /1g testis ($*10^6$ cell)	Number of 10 μm isolate cells /1g testis ($*10^6$ cell)	Viability (%)	Number of viable cells /1g testis ($*10^6$ cell)
L-15	10% DMSO	2.28 ± 0.10^{aA}	1.55 ± 0.10^{aA}	77.58 ± 1.28^{aA}	1.20 ± 0.08^{aA}
	10% PG	1.68 ± 0.14^{bA}	0.94 ± 0.09^{cA}	68.33 ± 0.98^{cdA}	0.64 ± 0.06^{cA}
	10% EG	1.47 ± 0.08^{cA}	0.79 ± 0.07^{dA}	67.83 ± 1.40^{cdA}	0.53 ± 0.03^{dA}
Mounib	10% DMSO	1.73 ± 0.07^{bA}	1.07 ± 0.04^{bA}	71.75 ± 1.29^{bA}	0.77 ± 0.04^{bA}
	10% PG	1.34 ± 0.09^{cA}	0.71 ± 0.04^{dA}	66.66 ± 1.43^{dA}	0.47 ± 0.04^{dA}
	10% EG	1.37 ± 0.06^{cA}	0.72 ± 0.05^{dA}	66.50 ± 1.76^{dA}	0.48 ± 0.05^{dA}
NAM	10% DMSO	1.42 ± 0.09^{cA}	0.76 ± 0.07^{dA}	69.16 ± 1.69^{cA}	0.53 ± 0.05^{dA}
	10% PG	1.36 ± 0.09^{cA}	0.69 ± 0.05^{dA}	67.25 ± 1.03^{cdA}	0.46 ± 0.03^{dA}
	10% EG	1.46 ± 0.06^{cA}	0.75 ± 0.07^{dA}	67.33 ± 1.32^{cdA}	0.50 ± 0.05^{dA}
Fresh		9.17 ± 0.46^{B}	3.08 ± 0.19^{B}	88.25 ± 0.88^{B}	2.73 ± 0.16^{B}
Negative		0.25±0.03	0.03±0.07	NS	NS

Table 4.2 Number of dissociated cells and their viability rates of frozen testes (Mean ± SD; N=6) (Cont.).

Extender	cryoprotectant	Number of total isolate cells/1g testis ($*10^6$ cell)	Number of 10 µm isolate cells /1g testis ($*10^6$ cell)	Viability (%)	Number of viable cells /1g testis ($*10^6$ cell)
Two-way analysis of variance (P<0.05)					
Extender		<0.001	<0.001	<0.001	0.026
Cryoprotectant		<0.001	<0.001	<0.001	0.002
Interaction		<0.001	<0.001	<0.001	0.001

Different letters indicate significant differences (P<0.05)., A indicates significant differences (P<0.05) between fresh and cryopreserved testes.

Table 4.3 Number of dissociated cells and their viability rates of frozen testes with varying concentration of cryoprotectant (DMSO) (Mean±SD; N=6).

Extender	cryoprotectant	Number of total isolate cells /1g testis (*106 cell)	Number of 10 µm isolate cells/1g testis (*106 cell)	Viability (%)	Number of viability cells/1g testis (*106 cell)
L-15	7.5% DMSO	1.32±0.05^{cA}	0.62±0.03^{cA}	74.50±0.63^{cA}	0.46±0.02^{cA}
	10% DMSO	2.35±0.03^{aA}	1.57±0.05^{aA}	78.16±0.98^{aA}	1.23±0.05^{aA}
	12.5% DMSO	1.83±0.03^{bA}	1.11±0.04^{bA}	76.33±1.32^{bA}	0.84±0.04^{bA}
	15% DMSO	1.37±0.04^{cA}	0.64±0.02^{cA}	74.25±0.58^{cA}	0.48±0.02^{cA}
Fresh		9.25±0.0^{B}	2.99±0.15^{B}	88.08±0.58^{B}	2.63±0.12^{B}
Negative		1.8±0.25	0.05±0.01	NS	NS

Different letters indicate significant differences (P<0.05)., A indicates significant differences (P<0.05) between fresh and cryopreserved testes.

Table 4.4 Number of dissociated cells and their viability rates of frozen testes with varying thawing condition (Mean±SD; N=6).

Temperature	Time	Number of total isolate cells /1g testis (*106 cell)	Number of 10 µm isolate cells /1g testis (*106 cell)	Viability (%)	Number of viable cells /1g testis (*106 cell)
10°C	4 min	1.64±0.09^{cA}	1.06±0.04^{cA}	76.9±1.3^{bA}	0.82±0.04^{cA}
	8 min	2.26±0.04^{aA}	1.68±0.05^{aA}	80.8±2.3^{aA}	1.37±0.07^{aA}
	10 min	2.06±0.04^{bA}	1.50±0.03^{bA}	78.5±0.6^{bA}	1.18±0.03^{bA}
28°C	4 min	1.56±0.06^{cA}	0.97±0.05^{dA}	72.5±0.6^{cA}	0.70±0.04^{dA}
	8 min	1.41±0.06^{dA}	0.82±0.02^{eA}	69.9±0.5^{dA}	0.58±0.01^{eA}
	10 min	0.95±0.07^{eA}	0.53±0.04^{fA}	67.8±0.9^{eA}	0.36±0.03^{fA}
Fresh		7.81±0.02^{B}	3.06±0.01^{B}	88.5±0.5^{B}	2.71±0.08^{B}
Negative		0.03±0.04	0.08±0.01	NS	NS
Two-way analysis of variance (P<0.05)					
Temperature		<0.001	<0.001	<0.001	<0.001
Time		<0.001	<0.001	<0.001	<0.001
Interaction		<0.001	<0.001	<0.001	<0.001

Different letters indicate significant differences (P<0.05)., A indicates significant differences (P<0.05) between fresh and cryopreserved testes.

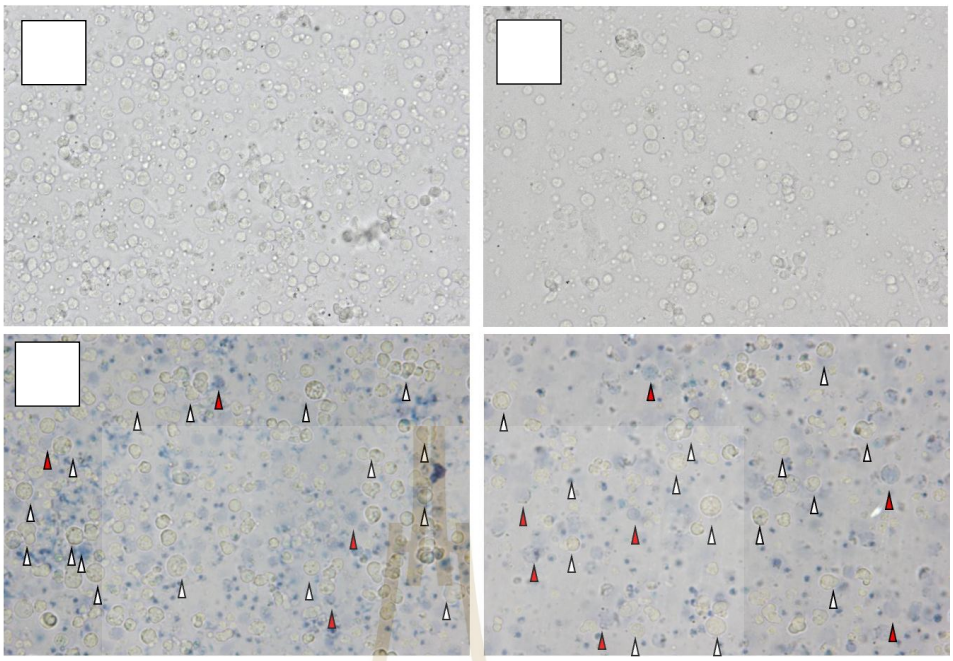

Figure 4.2 Spermatogonia cell in fresh testes (A-B) and cryopreserved cell (C-D) using trypan blue stained assessed (white arrowhead show viable cells and red arrowhead show dead cells) (Scale bar = 20 μm.).

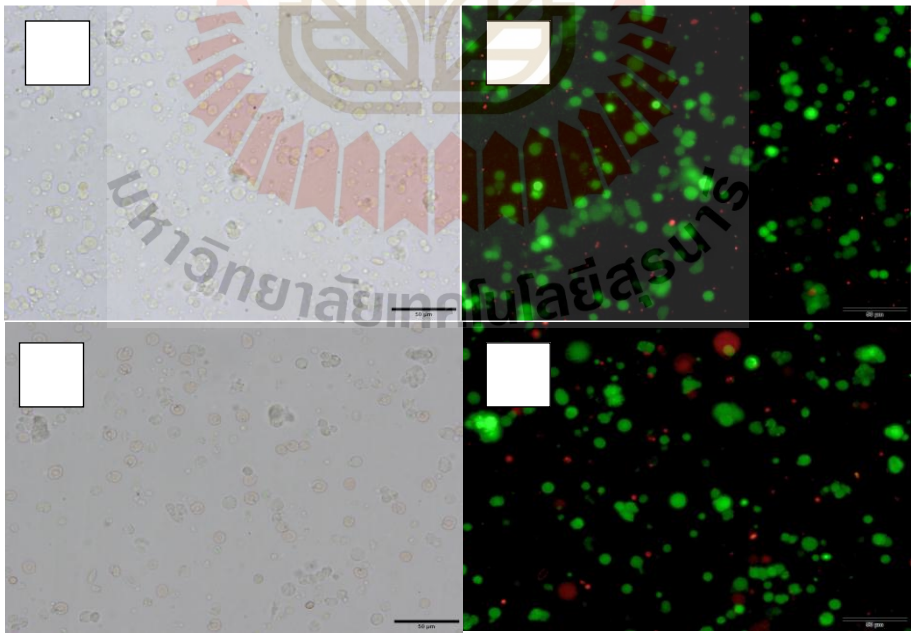

Figure 4.3 Spermatogonia cell in fresh testes (A-B) and cryopreserved cell (C-D) stained FDA and PI (white arrowhead show viable cells and red arrowhead show dead cells) (Scale bar = 20 μm.).

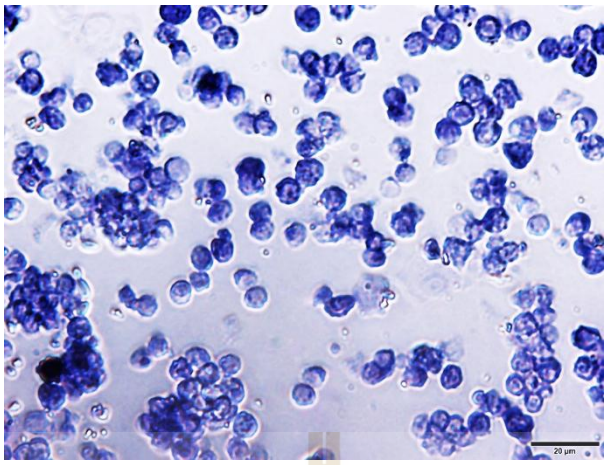

Figure 4.4 Spermatogonial cell obtained from fresh testes showed positive vasa expression (Scale bar = 20 µm.).

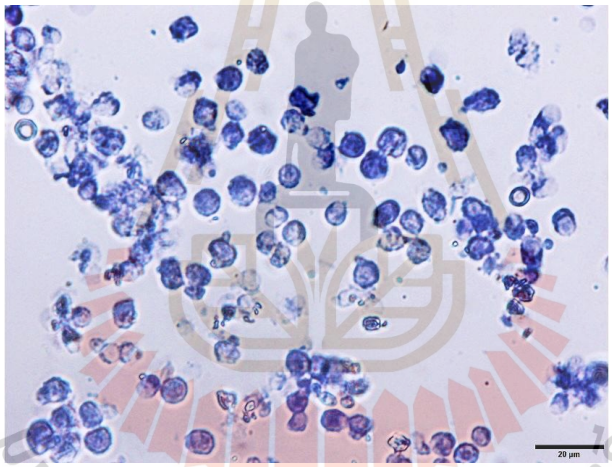

Figure 4.5 Spermatogonial cell obtained from frozen testes showed positive vasa expression (Scale bar = 20 µm.).

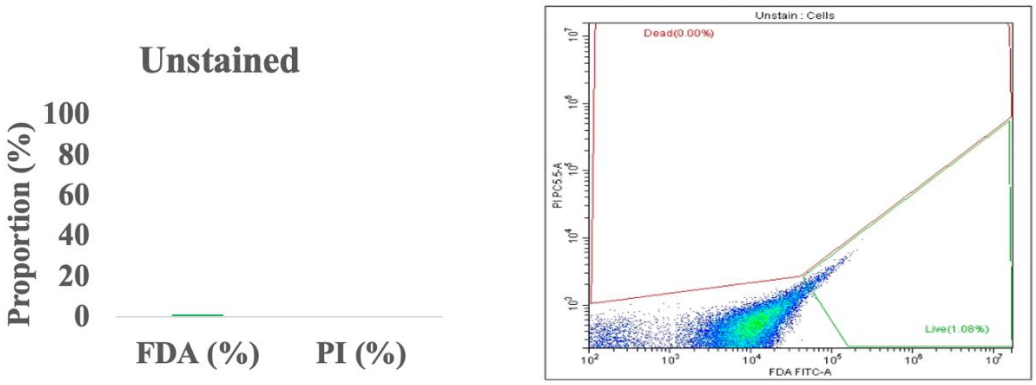

Figure 4.6 Flow cytometric analysis with permeable esterase substrate fluorescein diacetate (FDA) and the cell impermeable DNA stain propidium iodide (PI). Unstained cell used for control.

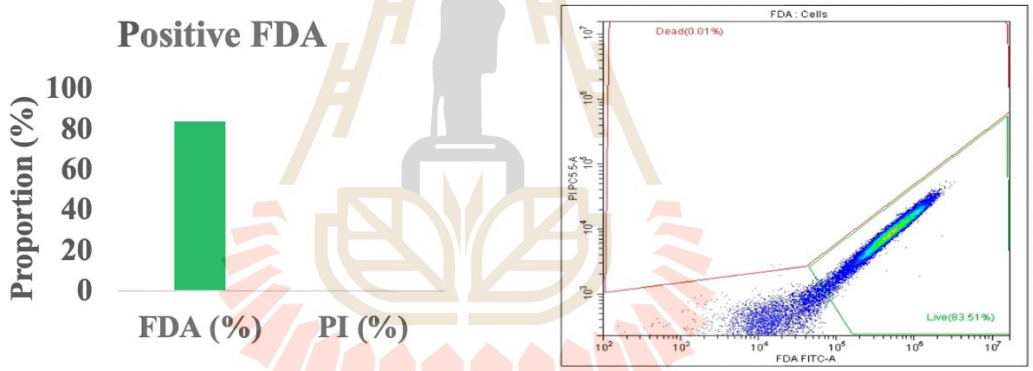

Figure 4.7 Flow cytometric analysis with permeable esterase substrate fluorescein diacetate (FDA) and the cell impermeable DNA stain propidium iodide (PI). Positive cell used for positive FDA in experiment.

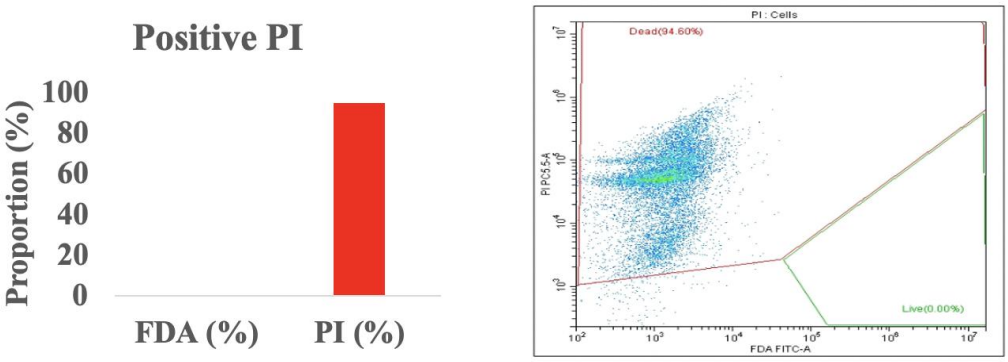

Figure 4.8 Flow cytometric analysis with permeable esterase substrate fluorescein diacetate (FDA) and the cell impermeable DNA stain propidium iodide (PI). Positive cell used for positive PI in experiment.

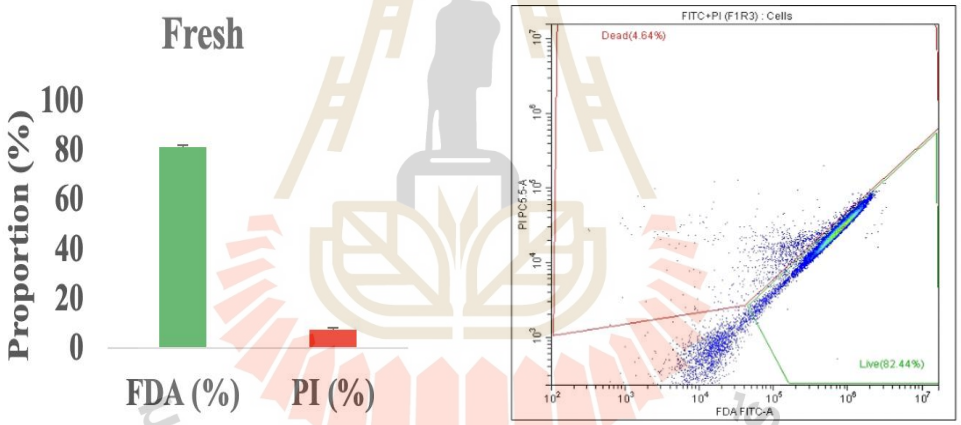

Figure 4.9 Flow cytometric analysis with permeable esterase substrate fluorescein diacetate (FDA) and the cell impermeable DNA stain propidium iodide (PI). Percentage of FDA and PI in Fresh spermatogonial cells.

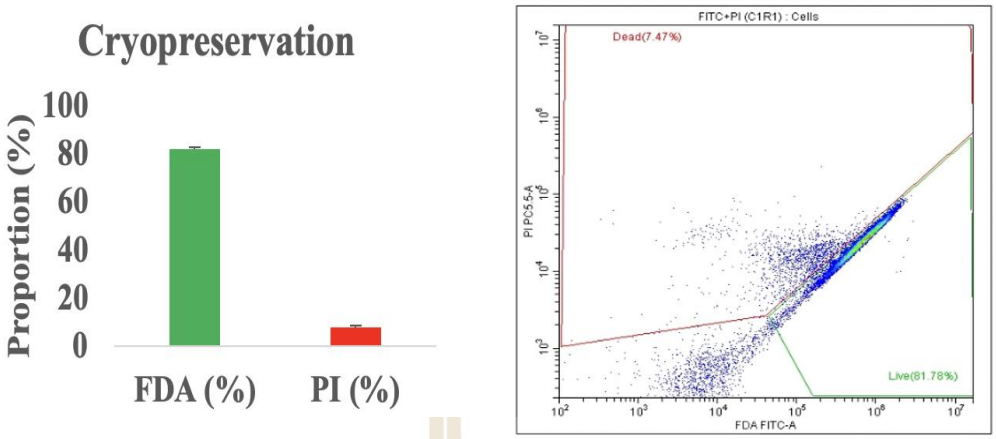

Figure 4.10 Flow cytometric analysis with permeable esterase substrate fluorescein diacetate (FDA) and the cell impermeable DNA stain propidium iodide (PI). Percentage of FDA and PI in cryopreserved spermatogonial cells.

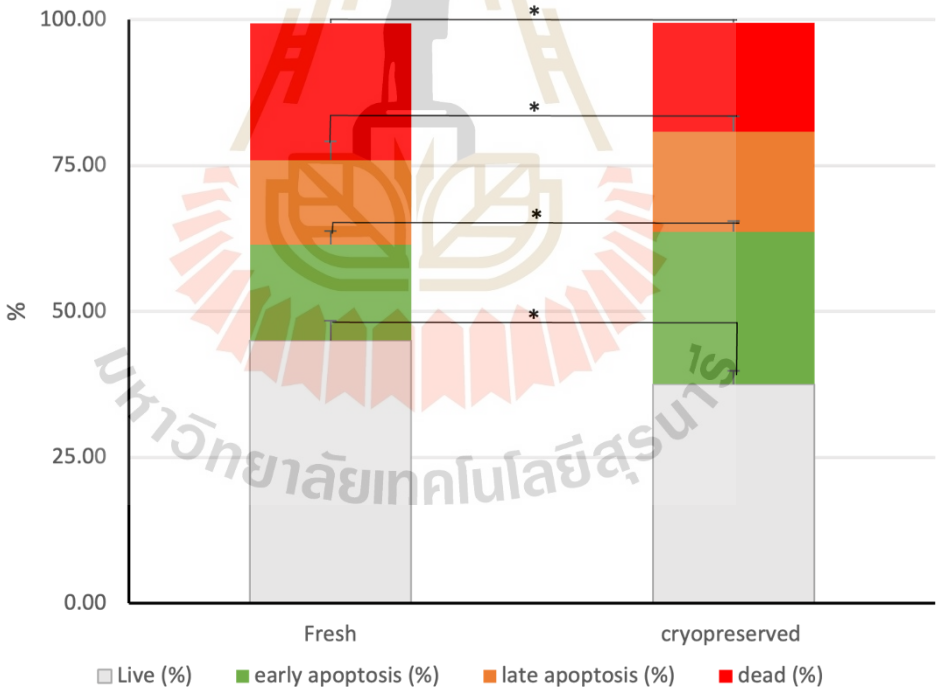

Figure 4.11 Apoptosis analysis used flow cytometric profile of dissociated cells labelled with Annexin V and PI included 4 stages.

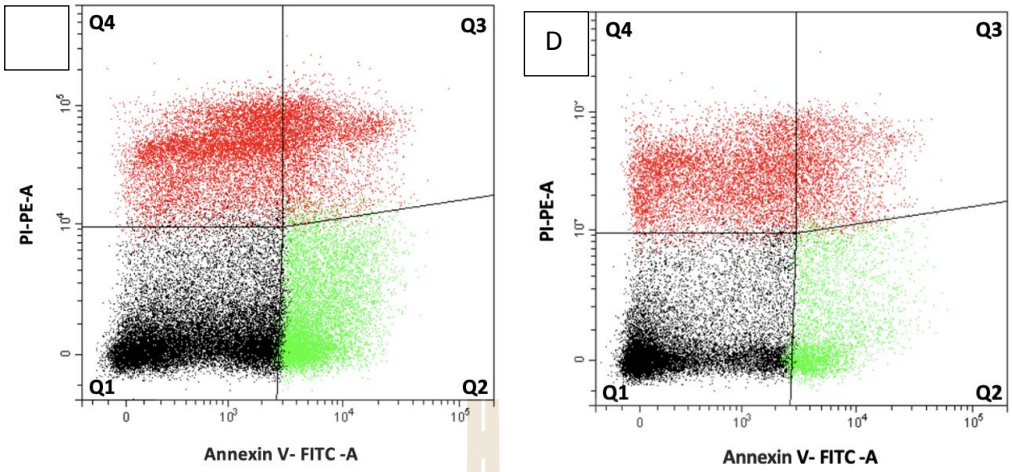

Figure 4.12 Apoptosis analysis used flow cytometric profile of dissociated cells labelled with Annexin V and PI included 4 stages (live cell, Q1, early apoptosis, Q2, late apoptosis, Q3, dead cell, Q4). Apoptosis in cryopreserved spermatogonial cell. (A) Fresh cell, (B) Cryopreserved cell.

4.3 Cryopreservation of testis in the Asian sea bass

To evaluate whether testicular cells prepared from cryopreserved testes could exhibit transplatability, in this study prepared the red fluorescent cryopreserved spermatogonial cell and used as donor cell for intraperitoneal microinjection into allogenic recipient (Figure 4.22). Survival rate of transplanted fry was 47.00+3.63% compared to control Uninjected fry (48.17+4.63%). Our results showed that while there was no red fluorescent cell colonized in the genital ridge of control un-injected fry (Figure 4.23), the red fluorescent cell obtained from cryopreserved testes was able to migrate and incorporated into the genital ridge of recipient fry (Figure 4.25 and 4.26). Note that the percentage of recipients have red fluorescent cell obtained from fresh testis colonized in genital ridge were 70.83+4.92% (data not shown). For cryopreserved testis, 52.50+5.24% of recipients fry showed incorporation of red fluorescent cell obtained from cryopreserved testis in genital ridge.

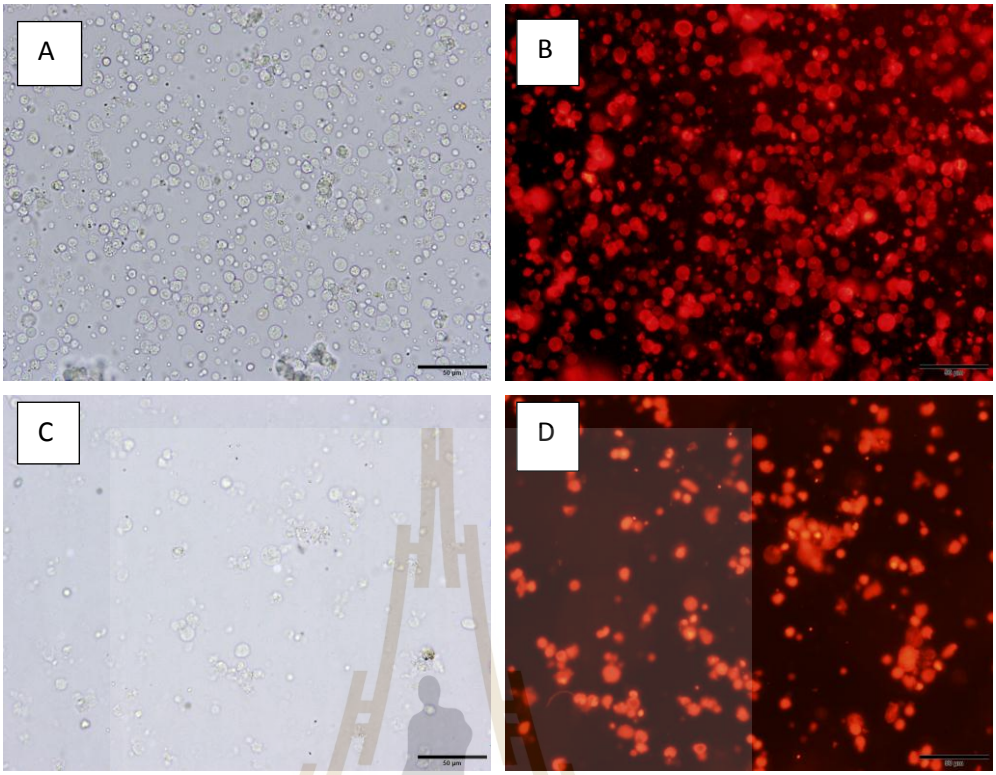

Figure 4.13 Spermatogonial cell stained PKH 26 in fresh testes (A-B) and post-thaw testes (C-D) (Scale bar = 20 μm.).

Figure 4.14 Intraperitoneally transplanted into allogenic recipient (arrowhead indicated peritoneal cavity and arrow indicated transplanted needle).

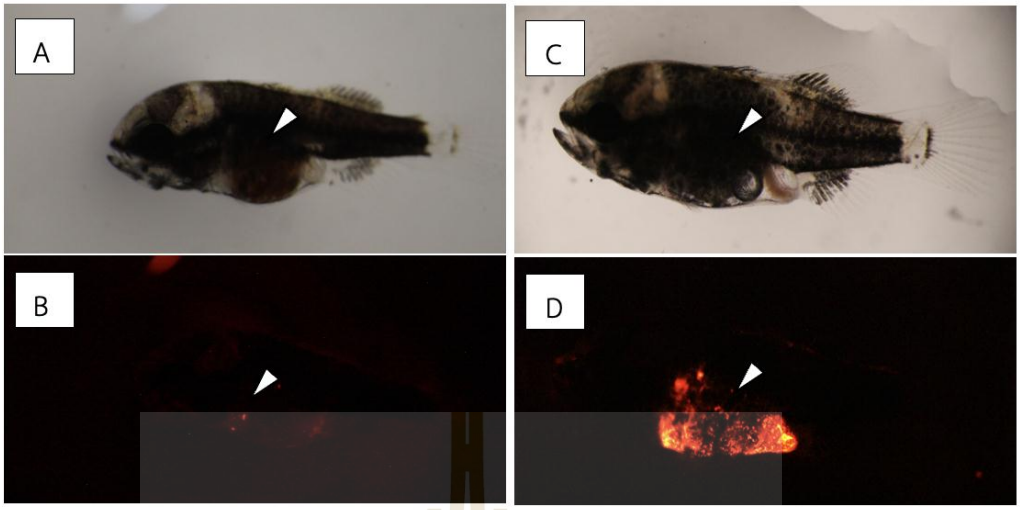

Figure 4.15 Peritoneal cavity of control un-injected fry (A-B) and the red fluorescent cell obtained from peritoneal cavity of transplanted fry (C-D).

Table 4.5 Survival rate of control, fresh and cryopreservation testes after 14 days of transplantation (dpt) **(**Mean±SD; N=6).

Replication	No of transplanted	No of survived	No of colonization (20 fish)	Survival rate	Colonizationlate
Fresh spermatogonia					
Transpanted 1	100	49	15	49	75
Transpanted 2	100	44	14	44	70
Transpanted 3	100	42	15	42	75
Transpanted 4	100	49	13	49	65
Transpanted 5	100	52	13	52	65
Transpanted 6	100	53	15	53	75
Average	100			48.17±4.36[b]	70.83±4.92[a]
Frozen spermatogonia					
Transpanted 1	100	47	9	47	45
Transpanted 2	100	41	12	41	60
Transpanted 3	100	45	11	45	55
Transpanted 4	100	48	10	48	50
Transpanted 5	100	50	11	50	55
Transpanted 6	100	51	10	51	50
Average	100	47.00	10.50	47.00±6.63[b]	52.50±5.24[b]

Table 4.5 Survival rate of control, fresh and cryopreservation testes after 14 days of transplantation (dpt) **(**Mean±SD; N=6**)** (Cont.).

Replication	No of transplanted	No of survived	No of colonization (20 fish)	Survival rate	Colonizationlate
Control					
control 1	100	55	-	53	-
control 2	100	52	-	55	-
control 3	100	56	-	50	-
control 4	100	51	-	54	-
control 5	100	56	-	56	-
control 6	100	50	-	57	-
Average	100	53.33	-	54.17±2.48[a]	-

Different letters indicate significant differences (P<0.05).

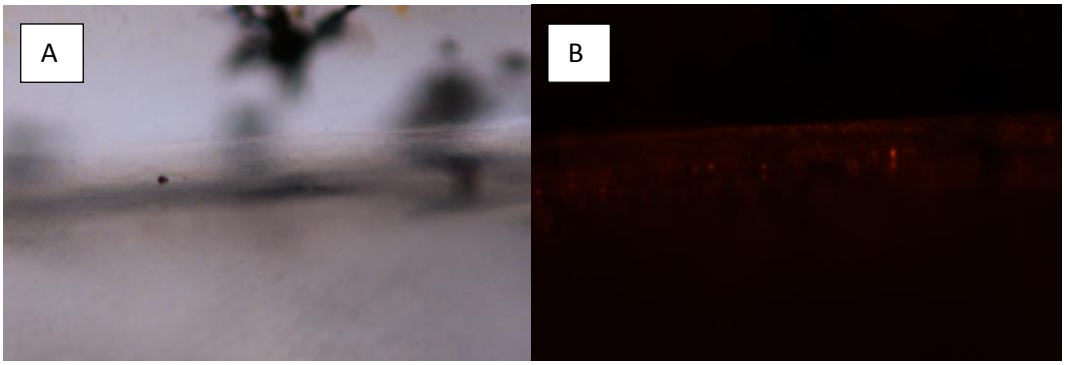

Figure 4.16 There was no red fluorescent cell colonized in the genital ridge of control un-injected fry.

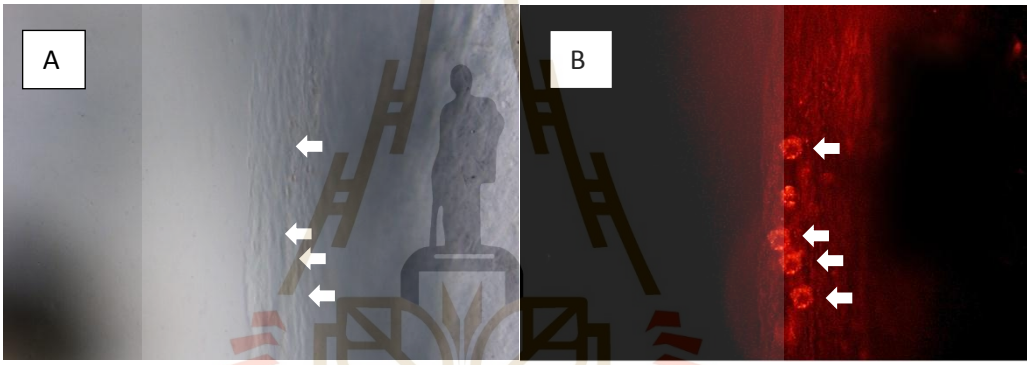

Figure 4.17 Red fluorescent cell colonized in the genital ridge of fresh testes injected fry.

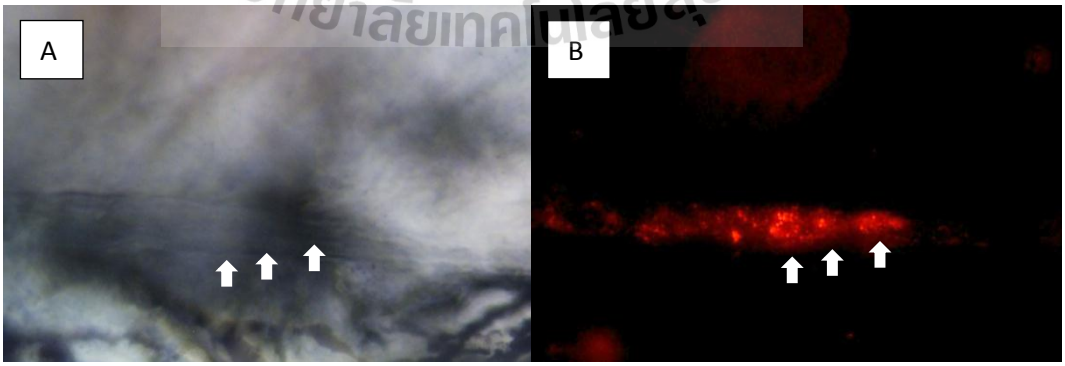

Figure 4.18 Red fluorescent cell colonized in the genital ridge of Cryopreservation testes injected fry.

CHAPTER V
DISCUSSION

Preservation of spermatogonia could be accomplish of whole testes by cryopreservation and dissociated spermatogonia, and whole testes after cryopreservation was proposed to be more applied than that of dissociated spermatogonia cells (Psenicka et al., 2016). In the present study, cryopreservation of whole testes was conducted to preserve spermatogonia cells. Moreover, cryopreservation of spermatogonial cells using cryopreserved whole testes were substantiated, and most of testes were achieve from immature fish. The immature testes were shown to contain spermatogonial in which the size was approximately 10 µm such as in Manchurian trout (*Brachymystax lenok*) (Lee and Yoshizaki, 2016), rainbow trout (*Oncorhynchus mykiss*) (Lee et al., 2013; Lee et al., 2016), common carp (*Cyprinus carpio*) (Franek et al., 2019), blue catfish (*Ictalurus furcatus*) (Abualreesh et al., 2020), Australian rainbowfish (*Melanotaenia fluviatilis*) (Rivers, et al., 2020) and blue catfish (*Ictalurus furcatus*) (Abualreesh et al., 2020). as part of this study, therefore, histological study was firstly performed to determine the size (and/or age) of The Asian seabass that their gonads contained high proportion of spermatogonial cells (~10 µm). Predictably the testes of immature Asian sea bass at size of 75-500 g (less than 1 year) contained high proportion of spermatogonial cells. To decrease fish number used in this study, the Asian sea bass at size 300-500 g were used through this experiment because of their largest testes size.

For cryopreservation of whole immature testes, several factors were investigated to have effects on viability of post-thaw viable spermatogonia including extender, permeating cryoprotectant and their concentrations and also thawing condition. Moreover, develop possibility method of farmed fish, the method of slow freezing rate -1°C/min was used for cryopreservation of whole immature testes in the Asian sea bass. Furthermore, the freezing rate -1°C/min was demonstrated to yield high viability rates of spermatogonia in frozen whole testes marine goby (Hagedorn et al., 2018), Siberian sturgeon (Psenicka et al., 2016), Manchurian tout (Lee and

Yoshizaki, 2016), zebrafish (Marinovic et al., 2019) common carp (Franek et al., 2019), rainbow fish (Rivers et al., 2020) and blue catfish (Abualreesh et al., 2020).It has been showed that cryopreservation medium and protocol for successfully cryopreserved whole testes are species-specific and variable. For optimization of slow-freezing protocol for testes tissue freezing of Asian seabass, suitable cryomedium containing extender, permeating cryoprotectants were decided. amongst tested extenders, L-15 was the best extender for freezing medium with DMSO. The Moubin's extender did not gave the highest viability rate though it was shown to be suitable for extender of frozen semen in the Asian sea bass (Zilli et al., 2012). Suggesting that extender might be tissue and/or cell specific. Indeed, not for the Moubin's extender, Non-Activating Medium (NAM) was used as suitable fish semen as well as the Asian sea bass semen (Martínez-Páramo et al., 2013).

Cryoprotectants, DMSO were suitable cryoprotectants while PG and EG were the poor-performing cryoprotectant for the whole testis cryopreservation in the Asian sea bass. Consequent, varying concentration of DMSO at 10 % was superior to that of DMSO at all concentrations to produce post-thaw viable cells. Contrary, DMSO with varying concentration was explained to be generally effective cryoprotectant in rainbow trout (Lee et al., 2013), marine goby (Hagedorn et al., 2018), zebrafish (Marinovic et al., 2019), common carp (Franek et al., 2019), rainbow fish (Rivers et al., 2020), and blue catfish (Abualreesh et al., 2020). Nevertheless, different cryoprotectants such as ethylene glycol and methanol were shown to be the best cryoprotectants for cryopreservation of testes in Siberian sturgeon and Manchurian trout, respectively (Psenicka et al., 2016; Lee and Yoshizaki, 2016). Overall, the suitable cryomedium for whole immature testes of the Asian sea bass were determined, and this freezing medium was furthermore used through this study to investigate thawing method. Optimization of thawing condition including thawing temperature and time is also important step to minimize cryoinjury caused by intracellular ice formation and attackable by cryoprotectant toxicity. In Manchurian trout, thawing temperature had significant effects of viability of testicular cell. Thawing temperature at 10–30°C had higher survival rate comparing with that of 40°C (Lee and Yoshizaki, 2016). In this study, thawing temperature at 10°C for 8 min resulted in highest viable spermatogonial cells. Hence, the optimization condition of

cryopreservation with slow-freezing method for whole testes in the Asian sea bass included cryomedium containing L-15 and 10% DMSO with thawing at 10°C for 8 min.

To validate viability rate of cryopreserved spermatogonia like cell, simultaneous staining of cellular permeable esterase substrate FDA and cellular impermeable DNA stain PI (FDA/PI) was used. The FDA/PI staining method was used to evaluate cell membrane integrity and viability in mammalian cells including isolated islet of Langerhans in pig and mouse (Boyd et al., 2008; Chandravanshi et al., 2014). Using FDA/PI staining in conjunction with flow cytometer, in this study investigated viability rates of the best condition of cryopreservation whole testes. Our results showed that the viability rates of both fresh and cryopreserved spermatogonia like cells were in the same trend of that using trypan blue staining. Moreover, investigation was performed to determine whether cryopreservation whole testes had effects on apoptosis. Destabilization of plasma membrane due to ice crystal formation, change in solute concentration and toxicity of cryoprotectant in slow- freezing method which affects cell survival rate. These conditions might affect translocation of PS from the intracellular to the outer leaflet, indicating an early sign of apoptosis. Late apoptosis was occurred when plasma membrane is weak and leaky. Subsequently, in necrotic and apoptotic cell death, disorder plasma membrane integrity leads to leakage of cellular organelle and contents. Effects of cryopreservation on apoptosis induction was demonstrated to induce apoptosis in several cryopreserved sperm, hepatocytes (Fu et al., 2001; Anzar et al., 2002; Vasicek et al., 2022). However, cryopreservation had no effects on apoptosis in peripheral blood monocellular cells (Riccio et al., 2002). In this experiment, using Annexin V/PI assay followed by flow cytometry, comparing to spermatogonial cell obtained from fresh testes and freezing testes increased early and late apoptosis in cryopreserved cells which might be at least in part a cause of lower viability rate. It was observed that enzymatic dissociated process also disrupted membrane integrity which resulted in necrotic cells in fresh testes.

To provide as a tool for restoration of the frozen cells, cryopreservation combination with germ cell transplantation technology is required. Cryopreserved spermatogonial cells were revealed to maintain transplantability in xenogenic germ cell transplantation. For instance, inter-specific germ cell transplantation was carried

out to restore cryopreserved spermatogonia cells of carp in goldfish recipient (Franek et al., 2019). In addition, long-term cryopreserved spermatogonia of rainbow trout was able to colonize in genital ridge of triploid recipient masu salmon (Lee et al., 2016). Moreover, using allogenic germ cell transplantation, cryopreserved spermatogonia exhibited transplantability characteristic in zebrafish (Marinovic et al., 2019), Manchurian trout (Lee and Yoshizaki, 2016) and rainbow trout (Lee et al., 2013). This study was performed allogenic germ cell transplantation to test whether the frozen spermatogonial cell could be used for germ cell transplantation. This study presented that although the incorporation rate was lower than that of fresh testes, the frozen spermatogonial cells obtained from cryopreserved testes could incorporated into the genital ridge of recipient fish, suggesting that. Therefore, although not totally like fresh testes, the spermatogonia cells isolated from cryopreservation of whole immature testes of the Asian sea bass retained their SSC and transplantability characteristics.

In conclusion, this study optimized cryopreservation for slow-freezing method including cryomedium containing extender and cryoprotectant and thawing process for whole testes in the Asian seabass. Apoptosis would be a significant cause leading to lower viability rate of cryopreserved cell. The spermatogonial cells isolated from cryopreserved testes retained spermatogonia stem cell characteristics and could be used for germ cell transplantation. Apoptosis would be a significant cause leading to lower viability rate of cryopreserved cell compared to fresh cell. The spermatogonial cells isolated from cryopreserved testes showed similar expression level of several gene maker of immature germ cell, suggesting its retaining of spermatogonia stem cell characteristics. These post-thaw spermatogonia also exhibited transability.

CHAPTER VI
CONCLUSION

6.1 The fish at 300-500 g BW was use throughout this study. Indeed, its testes contained spermatogonia like cell ~ 3.08 x 10^6 cell/g testes.

6.2 The suitable cryopreservation conditions for testes of the Asian seabass were cryomedium containing L-15 and 10% DMSO with thawing conditions at 10°C for 8 min which was used throughout this study.

6.3 Flow cytometric analysis with permeable esterase substrate fluorescein diacetate (FDA) and the cell impermeable DNA stain propidium iodide (PI)). The viability rate of spermatogonia from cryopreserved testes (81.63±0.92%) was no significant different of fresh testes (81.06±1.11%)

6.4 Apoptosis analysis showed early apoptotic cell in cryopreserved testes was significantly higher than that obtained from the fresh testes, demonstrating that post-thaw process induced apoptosis compared to fresh testes. This observation could suggest that apoptosis which is a process of programmed cell death occurred in cryopreservation of whole testes

6.5 In situ hybridization, spermatogonial cell obtained from both fresh and cryopreserved testes showed positive vasa expression, suggesting its spermatogonia stem cell characteristic.

6.6 There was no red fluorescent cell colonized in the genital ridge of control un-injected fry. The red fluorescent cell obtained from cryopreserved testes was able to migrate and incorporated into the genital ridge of recipient fry. Note that the percentage of recipients have red fluorescent cell obtained from fresh testis colonized in genital ridge were 70.83+4.92 %. For cryopreserved testis, 52.50+5.24 % of recipients fry showed incorporation of red fluorescent cell obtained from cryopreserved testis in genital ridge.

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APPENDIX

Chemical composition Leibovitz's L-15 Medium

(product No. A1323)

Composition (mg/L)

- INORGANIC SALTS	
Calcium chloride dehydrate	185.00
Magnesium chloride hexahydrate	200.00
Magnesium sulphate anhydrous	97.72
Potassium chloride	400.00
Potassium phosphate, monobasic	60.00
Sodium chloride	8,000.00
Sodium phosphate, dibasic anhydrous	190.12
- AMINO ACIDS	
DL-Alpha alanine	450.00
Glycine	200.00
L-Arginine (free base)	500.00
L-Asparagine	250.00
L-Cysteine (free base)	120.00
L-Glutamine	300.00
L-Histidine (free base)	250.00
L-Isoleucine	250.00
L-Leucine	125.00
L-Lysine hydrochloride	94.00
L-Methionine	75.00
L-Phenylalanine	125.00
L-Serine	200.00
L-Threonine	300.00
L-Tryptophan	20.00
L-Tyrosine Disodium Salt	276.16
L-Valine	100.00

- VITAMINS	
Choline chloride	1.00
D-Ca-Pantothenate	1.00
Folic acid	1.00
Nicotinamide	1.00
Pyridoxine hydrochloride	1.00
Riboflavin-5-phosphate, Na	0.10
Thiamine monophosphate	1.00
I-Inositol	2.00
- OTHERS	
D-Galactose	900.00
Phenol red Sodium Salt	11.00
Sodium pyruvate	55

BIOGRAPHY

Somkiat Sreebun born on 28 October 1995 in Bangbung, Chonburi, Thailand. In 2014 finished high school from Banbung Uttasahakamnukhro School, Bangbung, Chonburi. In 2019 graduated the Bachelor's degree in Aquatic Animal Production Technology, Faculty of Animal Sciences and Agricultural Technology, Silpakorn University, Phetchaburi. In 2019 began a master's studies in Biotechnology for Aquaculture, Institute of Agriculture Technology, Suranaree University of Technology, Nakhon Ratchasima, Thailand