Preliminary Study on Testicular Germ Cell Transplantation of Endemic Species *Oryzias celebensis*

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Abstract. The research has been conducted to study some technical steps for male germ-plasm from endemic fish species such as some species of *Oryzias* fish in Indonesia to preserve and propagate through germ cell transplantation technology. For preliminary research, the study was started with germ cell characterization of testes, cryopreservation of TGC and the transplantation of *Oryzias celebensis* as candidates for surrogate broodstock of *Oryzias* fish male germ plasm. The data analyzed included the potential number of TGC as donor, the viability of cryopreserved TGC in two types of cryoprotectants and the survival rate of *O.celebensis* larvae as recipient after transplantation. The result showed that the average amount of TGC yielded after dissociation was 131000 ± 31349 with 74.2 % viability of TGC each. Cryoprotectant 10% DMSO + glucose yielded higher viable of TGC. More than 80 % of *O.celebensis* larvae survived after transplantation. In conclusion, these preliminary data of *O.celebensis* as surrogate broodstock candidate will support the application of TGC transplantation technology in *Oryzias* endemic species.

1. Introduction

Genus *Oryzias* is one of the endemic fishes in South Sulawesi which some of them have been included in the red list of endangered fish. Overfishing so far has not been accompanied by efforts to the cultivation lead to extinction. Therefore, *Oryzias* fish conservation efforts as part of the diversity or biodiversity of fish, especially in South Sulawesi needs to be done. Recent studies have indicated that germ cell transplantation and cryopreservation technology have become technical breakthrough and prospect approach to solve the production of seed for commercially valuable fish, as well as threatened species.

Surrogate broodstock production through germ cell transplantation technology may eventually answer problems to the production of valuable commercially fish with long generation time. This technology has been applied in some fishes mainly in salmonids [1, 2] successfully produced surrogate broodstock and in tilapia resulted donor-derived spermatogenesis [3]. Male germ cell contain spermatogonia, the only cell responsible for generating offspring [4]. Spermatogonia donor cell transplanted into the peritoneal cavity of newly hatched embryo using microinjection technique. Transplanted spermatogonia will be migrated toward and colonized in the genital ridge of the recipient followed by proliferation and differentiation of donor derived germ cell into mature gametes in the
recipient. If spermatogonia of *Oryzias* fish could be successfully transplanted into short generation time fish then gametes of *Oryzias* fish could be easily and rapidly be produced. Therefore, germ cell transplantation technique is a potential breakthrough technique to preserve germ plasm of *Oryzias* fish.

Some basic techniques need to be established for the application of male germ cell transplantation in *Oryzias* fish including the preparation of male germ cell enriched in spermatogonia. To make the technique effective for transplantation, preparation of single germ cell suspension from a solid tissue is of critical importance. In general, it is important to maximize the number of viable cell, disperse clump of cells and minimize the reaggregation. There are six variable conditions should be well defined to get suitable results, those are dissociation medium, type of enzyme(s), enzyme preparation, concentration(s) of enzyme(s), temperature and incubation times.

Cryopreservation is a technique of storage or preservation of animal cells, plants or other genetic material (including semen and oocytes) in a frozen state by reduction of metabolic activity without affecting the organelles inside cells that the function of physiology, biology and morphology remain. In germ cell transplantation technology, cryopreservation is basic technique that should be done to synchronize availability of recipient and donor cell. The successfull of cryopreservation technique is usually determined by several factors, including the type of extender solution used. Currently there are sperms of ± 200 species of fish in the world successfully preserved using the techniques of cryopreservation, including carp (*Cyprinus carpio*) and Atlantic salmon (*Salmo salar*). However, none of them is found for reference of the cryopreservation done on celebensis medaka fish neither on cryopreservation of spermatogonial. Based on this, the research on cell cryopreservation of medaka fish germ cell needs to be done and as a treatment in this study will be observed the influence of some kind of solution extenders.

## 2. Materials And Methods

### 2.1 Collection of testes

Testes were collected from adult *Oryzias* fish (4 cm) that were obtained from Bantimurung river, Maros, South Sulawesi. Testes tissues for histological analysis (n=4) were fixed immediately after collection. For the dissociation of germ cell, collected testes were washed twice with Phosphate Buffer Saline (PBS) pH=8.2 (Gibco). All connective tissue at posterior and anterior ends, blood capillary and fat attached to gonad were removed from the collected testes and then testes were cut into pieces of 20-30 mg each for dissociation analysis.

### 2.2 Germ cell morphological analysis

Four testicular samples were fixed in Bouin fixative for 24 hours, dehydrated along an ascending series of ethanol according to [7], embedded in paraffin, and sectioned (5µm thickness). The block of tissue were sliced serially, and to avoids counting the same cell twice, 6 sections of 5 µm thickness per fish were taken every 35 µm sections. The sections were stained with Hematoxilin-Eosin for microscopic observation under the light microscope. Photographs were taken with an automatic Nikon camera. Germ cell staging was refers to [8]. For quantification of diameter and morphological character of germ cell type, three fields of view per section were selected at random from the middle portion of the testis as much 30 germ cell each type. Additionally, six cysts per type of spermatogonia were analyzed to obtain the average number of each type of spermatogonia per cyst.

### 2.3 Dissociation of germ cell

Testes were minced with scissors and incubated in dissociation medium at room temperature. For the purpose of defining the effect of dissociaton medium against the number and viability of spermatogonia germ cell, testes were incubated in dissociation medium. Dissociation medium contain PBS (Gibco) containing 0.5% trypsin and 30 µl of 10 IU/µl DNase (Sigma) in 1 ml PBS supplemented...
with fetal bovine serum (Sigma), Ca\textsuperscript{2+} (1mM) and 25 mM HEPES (Sigma). To obtain optimal yield of viable cell, testes were incubated in dissociation medium for two hours. During incubation, the cell suspension was pipetting gently every 30 minutes to dissociate germ cell tissue. Then, cell suspension was filtered through a 35 µm pore-size nylon screen to eliminate non-dissociated cell clumps and washed twice with PBS. The number and viability of spermatogonia were analyzed using CX 10 microscope and haemocytometer. Viability of the cells was analysed using the trypan blue dye exclusion test (1:1). Blue cell indicated damaged or death cell, while normal cell was transparant.

2.4 Cryopreservation of testicular cells
Cryopreservation method used in this study refers to the method. The cell suspension was diluted gametes dissociation results using extender solution with a ratio of 1:9. A total of 10 mL of testicular cells added to a solution of 90 mL extender. In this study, we used two different extender solution in 10% DMSO cryoprotectant : 5% glucose and Ringer solution. Before the suspension was added cryoprotectants testicular cells, testicular cells drops pipette and cell concentration is calculated in advance. Furthermore testicular cell suspension in cryoprotectants immediately put in a 10 mL size straw and put in cryotube. Cryotube left not closed tightly and put in bicell biofreezing vessel. Before freezing, gamete cells in Biocell containers are stored beforehand in the refrigerator -80°C for 20 hours. After that, cryotube containing testicular cells be closed tightly and put in a tube canister and stored in liquid nitrogen. Straw was stored in liquid N\textsubscript{2} containers for more than one week. To analyze testicular cell viability after cryopreservation, thawing process is done. Thawing carried out by dipping the straw into the water with temperature 30-40°C for 10-15 minutes. The testicular cells then ready to be observed using trypan blue vital staining.

2.5 Data analysis
Testes samples from 10 Oryzias fish were used for tissue dissociation and four testes for histological examination. Characterization of germ cell morphology was presented descriptively. Parameters for the testes dissociation were number and viability of spermatogonia and presented as mean.

3. Results and Discussion
3.1 Result
3.1.1 Morphological characteristic of Celebes Oryzias fish male germ cell. The testes of Oryzias fish form bilobed organ, with the right lobe slightly thinner and thinner than the left one (figure 1A). Cross section of seminiferous tubules at the middle part of Oryzias fish testes was depicted in Figure 1B. Histological analysis showed that all testes examined were producing germ cell at all the stages of development. Testes of Oryzias fish were unrestricted tubular in which of spermatogonia were distributed along the lengths of Oryzias fish testis tubules (figure 1B).
Figure 1 A. *Oryzias celebensis* fish testes. B. Histological structure of male germ cell of *Oryzias celebensis*. SSC = spermatogonial stem cell, SpA = Type A spermatogonia, SpB = Type B spermatogonia, Spc = spermatocyte, Spd = spermatid, Spz = spermatozoa.

Morphological characterization of germ cells were determined based on some criteria such as cell and nuclear size, nuclear morphology, topological organization of germ cell and cell number per cyst. Germ cell staging identified in *Oryzias* fish was as follows: Primary A spermatogonium (stem cell spermatogonium), A spermatogonia, transitional spermatogonium, B spermatogonium, primary spermatocyte, secondary spermatocyte, spermatid and spermatozoa. All proliferation stages between late A spermatogonia and early B spermatogonia were categorized as transitional spermatogonia. Thus, these morphological analysis allowed us to determine that there were three type of spermatogonia in immature 4 mm TL Of *Oryzias* fish. Spermatogonial stem cells were the largest single spermatogonia mostly located close to the basement membrane of the germinal epithelium. The nuclear membrane had an irregular outline, some of which remained unclear membrane as well. On the other hand, A spermatogonia were smaller in size, surrounded by Sertoli cells, forming 2-4 germ cell in a cyst located close to the lumina of the tubules. Morphologically, spermatogonial stem cell and some of type A spermatogonia were ellipsoid. The type B spermatogonia were smaller than A spermatogonia with the number of germ cells inside the cyst increases (18-48 germ cells within cyst).

3.1.2 Cell viability of testicular germ cell before and after cryopreservation. Observations were made in cells from three different treatment. Sample 1 was added to a solution of the extender in the form of glucose and DMSO as cryoprotectants, while sample 2 was added to a solution of the extender ringer and DMSO, and samples to 3 as a control is given only cryoprotectant DMSO alone without extenders. The results obtained from 3. The treatment of post-cryopreservation can be seen in Table 1. The average yield testing I and II showed that glucose extender is a solution that is more effective than the use of Ringer's solution and DMSO alone. In Table 1 are shown that the average yield of cell viability were using DMSO + extenders glucose after cryopreservation higher than the results of two other treatments, namely 1.964% on the replay I and 2.248 in replicates II, while the average viability of cells using extenders ringer and without extender solution are in the same position on the replay I is 0% and the second replay respectively 0.449% and 0.899%.
Table 1. Number of spermatogonia before and after cryopreservation with different extender.

| Sample | Extender | Viability before cryopreservation (%) | Viability after cryopreservation (%) |
|--------|----------|--------------------------------------|-------------------------------------|
| I      | 1 Ringer | 79.28                                | 0                                   |
|        | 2 Glukosa 5% | 79.83                                | 1.96                                |
|        | 3 DMSO    | 79.83                                | 0                                   |
| II     | 1 Ringer  | 64.39                                | 0.45                                |
|        | 2 Glukosa 5% | 64.39                                | 2.25                                |
|        | 3 DMSO    | 64.39                                | 0.89                                |

3.2 Discussion

Spermatogonia are stem cells that can restock their own population and simultaneously producing sperm on a renewable basic. This fact has become a benefit for technical application of assisted reproduction based on spermatogonia such as transplantation, cell culture or preservation of endangered and genetically valuable animals. This research is a first step prior to the effort to make surrogate broodstock using *Oryzias* fish as donor and transplantation technique assay. One of major limitation to the transplant procedure is that the donor germ cell mixture contained an unknown number of spermatogonia [9].

To increase the efficiency of transplantation, the percentage of spermatogonia with stem characteristic (Undifferentiated spermatogonia including spermatogonia stem cell and A spermatogonia) should be increased [10]. One of first successful studies increased the relative number of spermatogonia colonized in recipient by using testicular cell that were selected based on the ability of the cell to interact with the basement membrane of the seminiferous tubules [11]. In this research we just observed the type of testicular cells including type of spermatogonia morphologically using histological approach. Moreover, for viability assay, we just used trypan blue vital staining day to characterized normal viable cells. Although those technique approach had major limited information, but it helped us to prepare mixed germ cell enriched high number and viable spermatogonia for *Oryzias* fish transplantation assays.

Prior to dissociation of testes, we studied the morphological structure of testes and male germ cell type in *Oryzias* fish. Testicular parenchyma of *Oryzias* fish show typical organization as most of fish with two major component, the tubular and intertubular compartment. Similar to vertebrate, clearly it has been described that the tubular compartment organized in testes comprising the seminiferous epithelium, where the Sertoli/germ cells units called cysts are located, the tunica propria composed of basal lamina and peritubular myoid cells, and the tubular lumen filled with fluid secreted by Sertoli cells. While the intertubular compartment containing Leydig cells, connective tissue cells, and blood vessels [12-14]. The seminiferous tubules contain spermatogenic cysts, a group of synchronously developing germ cells that enveloped by sertoli cells, which are derived in a clonal manner from one stem cell or A spermatogonia in which interconnected by cytoplasmic bridge [3, 15].

The morphological characteristic of germ cell types observed for *Oryzias* fish was in general similar to most species of teleost fish that has been described previously [16, 17]. With histological approach, we have analyzed morphological features of *Oryzias* fish germ cell type based on nuclear and cytoplasm morphology and the number of germ cell per cysyte. Only three generations of spermatogonia could be classified in this research descriptively as 5 µm histological sections were disable to describe the nuclear morphology in clear performance. A spermatogonia in *Oryzias* fish were found at random sites along the seminiferous tubule throughout the testis. This type of germ cell organization has been classified as unrestricted type, a primitive character that appears to exist in a number of teleost fish orders such as cypriniformes, perciformes and others [18, 19].

Since each species has own anatomical characteristics including topological position of germ cell type, the relative amount of connective tissue between tubules, lobulation, the ease of removing tunica, need to be isolated then the protocol for the preparation of donor cell are species specific [20]. For
these reasons, we compared two protocols, those of modified protocol from [21] and modified protocol from [3, 22], the latter of which proved more efficient in Oryzias fish. However, 2 mechanical steps were required before tests exposure to digestive enzymes: first, removal of testicular blood vessels to eliminate the contamination of blood cell. Second, minced process of testes and gently pipetting of cell suspension provide an effective and fast enzymatic digestion, however being careful not to cause damage by squeezing the testes or by roughly pipetting. According to our observation, these mechanical steps could become critical point to get high number of spermatogonia yielded.

In conclusion, the dissociation of Oryzias celebensis testes yielded sufficient number of spermatogonia as donor cells for germ cell transplantation and cryopreservation still given a small number of donor cells available for germ cell transplantation.

Acknowledgements
We thank Prof Yusuke Naruse of The National Institute For Basic Biology, Okazaki, Japan for the assistance during training of 8th NIBB Practical Course for laboratory animals in his laboratory. We thank to Hasanuddin University for research grant. We also thank Brackish Water Fish Research Centre of Maros for some equipment and support of Oryzias fish dissociation which was performed in the laboratorium. Thank to Pharmacology Laboratorium of Pharmacy Faculty, UNHAS for allowing using liquid N2 containers.

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