# Donor preparation for germ cell transplantation in giant gouramy: the viability of spermatogonia isolated from giant gouramy cold preserved testis

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**Abstract.** The recent study has been conducted to develop testicular germ cell (TGC) transplantation as a tool for preservation and propagation of male germ-plasm from endangered fish species. In practice of TGC transplantation, recipient and donor cell may not be immediately available at the same time whereas the testis can not be survive longer when it is outside of the body. Therefore, preservation of testis tissue may be required before transplantation. The research was conducted to evaluate the viability of spermatogonia isolated from short term preserved testis. Testis was preserved in physiological NaCl solution at 4 °C for 6, 12, 24 and 48 hours. Testis were dissociated in 0.5 % trypsin and 3% DNase 10 IU/µL in PBS (phosphate buffered solution) complemented with 5% FBS ( fetal bovine serum), 25 mM HEPES and 1mM $CaCl_2$ to obtain testicular germ cell suspension. The testicular germ cells isolated from 24 and 48 hours preservation were performed in trypan blue staining dye (1:1) and the viability of spermatogonia were observed under microscope. The results showed that the viability of spermatogonia started to decrease significantly in 12 hours preservation ($P<0.05$) and up to 48 hours preservation, cell viability was as high as 54,48±8,33%. In conclusion, preserved testicular tissue at 4°C still produced viable spermatogonia that are allowed to use as the source of donor cell for testicular germ cell transplantation of giant gourami.
**Keywords**: preservation, spermatogonia, dissociation, giant gourami, viability

## Introduction

Germ cell transplantation technology to produce surrogate broodstock may eventually be one of the alternatives in facilitating the production of commercially valuable fish with long generation time. This technology was applied successfully to some fish species resulted in donor-derived spermatogenesis in tilapia (Lacerda *et al*., 2008) and in gametes production by the recipient animal in salmonids (Takeuchi *et al*., 2004; Okutsu *et al*., 2006). Male germ cell contains spermatogonia, the only cell responsible for generating offspring (Brinster & Avarbock, 1994). Male germ cell (as the donor cell) transplanted using microinjection technique into the peritoneal cavity of newly hatched embryo will migrate toward and form colony in the genital ridge of the recipient. Furthermore, donor-derived germ cell proliferated and differentiated into mature gametes in the recipient. If spermatogonia of giant gouramy were successfully transplanted into short generation time fish then gametes of giant gouramy could be easily and rapidly be produced. Therefore, germ cell transplantation technique does have the potential as a breakthrough technique to ensure sustainable seed production in giant gouramy culture.

On the application of transplantation techniques, synchronizing the availability of donor cells with recipient was being a big problem. Sometimes the cell or tissue donor is available but not ready for transplant recipients yet. In the meantime, if the donor is in the form of the testis after testicular tissue removed from the body of the fish will be at risk of damage if not immediately processed. Therefore, It is required storage techniques (preservation) to avoid damage to the cells prior to transplantation of gametes in the testis and to increase the survival rate of gametes as well.

There are two kinds of techniques that preserve long-term storage at temperatures below 0 ° C storage and preservation of short-term storage temperatures above 0 ° C (Browne et al. 2001). At some higher vertebrates, cryopreservation of testicular with uneven maturity level sometimes decrease cell viability, especially for cell spermatogonia or PGC (Jahnukainen et al., 2006, Ehmcke & Schlatt 2008). Cryopreservation of rainbow trout testicular germ cells produced the highest cell viability of approximately 50%, 40% less than control or without cryopreservation (Kobayashi et al. 2007). The effects caused by the cryopreservation technique is said to be inefficient for short-term storage (Jahnukainen et al. 2006). In this reasearch, we tried to perform short cold preservation of giant gouramy testis as a donor at 4 ° C . This type of preservation had not been done in any fish at all. In vertebrate animals, Eriani et al. (2008) performed preservation of ductus deferens and the epididymis cats at 4 ° C, and resulted the male gamete cells could still be alive for up to 6

days. Perhaps, this short cold preservation in physiological solution at 4 ° C could save the germ plasm during transportation as well.

## Materials and Methods

Five pairs of testis isolated from carp-sized adult male 700-800 g. Each testis put in physiological NaCl solution 0.7% sterile petri dish and preserved at 4 ° C with storage period 0, 6, 12, 24 and 48 hours each. Physiological solution of NaCl 0.7% was previously given antibiotics gentamycin 1.25 µL / mL. After the storage period was completed, the next testis removed from the cooler and dissociated according to methods of Andriani *et al*. (2010). As much as ± 20 mg of each testis was dissociated in 0.5 % trypsin and 3% DNase 10 IU/µL in PBS (phosphate buffered solution) complemented with 5% FBS ( fetal bovine serum), 25 mM HEPES and 1mM $CaCl_2$. Cell suspensions were washed with PBS 2 times to remove trypsin activity. Parameter measured was the viability of spermatogonia. A total of 10 µL of 1 mL germ cell suspension after dissociation was stained with 0.4% trypan blue (1:1). Death cells would stain with trypan blue so it looked like blue, while the viable cells would still look transparent. The total number of spermatogonia and death spermatogonia counted using a hemocytometer under a microscope. Each treatment was repeated three times. All qualitative data are presented descriptively, whereas quantitative data in the form of the value being tested statistically using ANOVA (analysis of variance), followed by Duncan's multiple range test trials to determine the significant difference between treatments. Analysis using SPSS 17.0 for Windows and MS Office Excel 2007. The difference in morphological characters tested descriptively.

## Results and Dicussion

The viability of spermatogonia from giant gouramy testis after preservation were shown in Table 1. The result showed that cold preservation (4 ° C) of testicular tissue (testis) in physiological saline solution affected spermatogonia viability significantly ($P < 0.05$). In the previous study, germ cell staging in immature giant gouramy weighed of 700-900 gram had been identified. Spermatogonia was characterized by larger cell with diameter more than 8.88±1.41 µm. The type of giant gouramy spermatogonia had been clearly identified as follows : Primary A spermatogonium (spermatogonial stem cell), A spermatogonium (SpA), transitional spermatogonium (SpT), B spermatogonium (SpB). Spermatogonial stem cells were the largest single spermatogonia with a cytoplasmic diameter of 18.63±1.92 µm and a nuclear diameter of 8.79 ±1.16 µm, and were mostly located close to the basement membrane of the germinal epithelium. Histologically, the nuclear membrane had an irregular outline, some of which remained unclear membrane as well (Andriani *et al*. 2010). However, the cell nuclear characteristic could not be identified from the germ cell suspension after dissociation. From this technique, the only way to identify spermatogonia was from the diameter of the cells as described in Figure 1.

Table 1 Number and viability of spermatogonia of giant gouramy testis at different period of cold preservation

| Preservation periods (hours) | The average no. of spermatogonia/mg testis | The spermatogonia viability (%) |
|---|---|---|
| 0 | 31,407 ± 8,668 | 96.77 ± 3.23[a] |
| 6 | 43,152 ± 2,240 | 88.37 ± 3.79[a] |
| 12 | 30,504 ± 1,997 | 77.70 ± 3.01[b] |
| 24 | 11,365 ± 3,201 | 74.30 ± 5.41[b] |
| 48 | 19,755 ± 12,102 | 54.48 ± 8.33[c] |

Values on in each column with same superscripts are not significantly different (P>0.05).

The viability started to decrease below 80% up to 12 hours preservation periods. Up to 48 hours preservation period, the viability dropped dramatically resulting nearly half of spermatogonia underwent cells death characterized by blue cells (Figure 1).

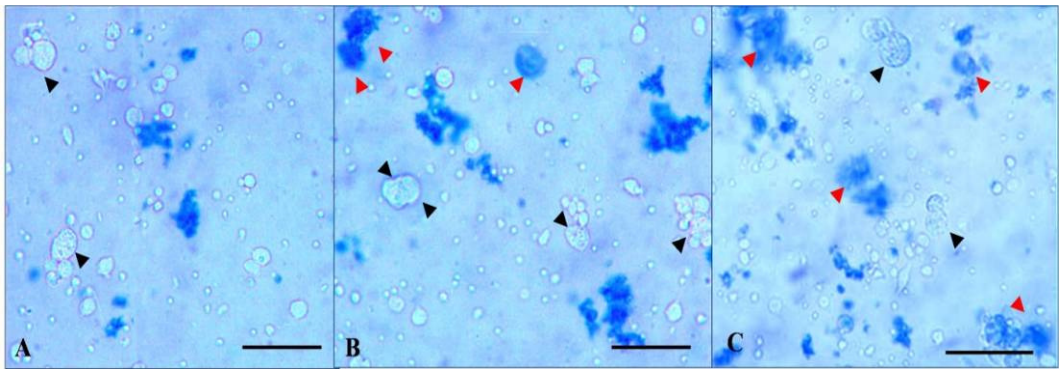


Figure 1. Giant gouramy testicular germ cell suspension after dissociation with donor source from testis without preservation(A), 24 hours testis preservation (B) and 48 hours testis preservation (C). Red arrowhead indicated a dead cell spermatogonia stained by trypan blue, while the black arrowheads indicated viable spermatogonia. Scale: 50 µm.

Preservation maintain and keep the material from any damage. Preservation can be either storage at low temperatures using chemicals. Preservation in the form of reduced temperature above freezing temperature and below body temperature can decrease metabolic activity, the need for oxygen, energy consumption, and hence it can prolong the preservation of cell viability (Honaramooz & Yang 2011). However, if the cooling is done too long it will destroy the balance and cellular homeostasis causing cells death. Generally, short-term storage temperature is the temperature of 4 $^{o}$C refrigerator.

The imbalance in the cell is also influenced by the role of reactive oxygen species (ROS), namely oxidative agents in the results of the derived category of free radicals of oxygen metabolism during the process of cellular respiration takes place (Sikka 1996). The product is in the form of compounds ROS free radicals such as O2-, H2O2, OH-can reduce cell viability (Aitken & Baker, 2006). During the process of preservation of organ or tissue, cell metabolism continues to live with the oxidation process. If the product of ROS in cells is in uncontrolled conditions, it will cause a negative effect on the cells.

Cold preservation technique is a short-term preservation techniques. This technique is very easy to apply in the field because it only needs the cooler (cool box) and a physiological solution. Physiological solution such as NaCl, PBS can also be a medium buffer and maintain the physiological pH (7.2 to 7.6) as well as providing an ionic liquid environment for cell metabolism (Daniel, 1971). With this cold preservation technique, testicular tissue from death fish will also potentially be saved and used as a source of donor so that the problem regarding the availability of donor cells that have been the limiting factor in transplantation activities can also be resolved. Preservation techniques can also contribute to efforts to save the fish gamete cells that are endangered may be found away from the location of the laboratory.

## Conclusions

Testis of giant gouramy could preserved at 4 $^{o}$C. Cell viability decreased to 55% after preservation for 48 hours allowing to use as donor for giant gouramy germ cell transplantation.

## Acknowledgements

The authors would like to thank Prof. Goro Yoshizaki of The Univesity of Marine Science and Technology (TUMSAT) Tokyo, Japan for his assitance during the Training of Dissociation and Transplantation at his laboratory as part of SANDWICH program (2008-2009) and supported by the General Directorate of Higher Education, Indonesia. The authors would also like to thank the Freshwater Aquaculture Development Centre in Sukabumi for providing materials and support of giant gouramy dissociation.

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