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Doctoral Dissertation

DEVELOPMENT OF GERM CELL TRANSPLANTATION SYSTEM USING POSTMORTEM RAINBOW TROUT AS A DONOR

September 2021

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Abstract

The captive breeding of fish has been conducting widely for the conservation and aquacultural purposes. Indeed, large number of research institutions and aquaculture farms have been maintaining valuable fish strains carrying desirable genetic traits for aquaculture or facing extinction. However, in general, captive breeding is always accompanied with potential risks of pathogen infection or facility accidents such as suspension of water supply and air pump or heater breakage. When valuable fish kept in captivity die by these potential risks, it is impossible to maintain their genetic resources anymore. However, if the gonads from the dead fish were applicable to be used for germ cell transplantation, it would be possible to preserve their genetic resources from surrogate broodstock. Since in general, germ cells from freshly sacrificed fish are used as donor in the germ cell transplantation, if viable germ cells can be retrieved from postmortem fish for a certain period, the genetic resources of postmortem fish may have possibility to be preserved by inheriting to next generation. In order to examine the feasibility of this scenario, in this study, we investigated the viability and transplantability of germ cells obtained from postmortem rainbow trout (Oncorhynchus mykiss). Nine-month-old vasa-Gfp male rainbow trout were killed by high dose of anesthesia and left in 10.5℃ of flowing water for 0, 6, 12 and 24 hours. ATP concentration and rigor index were measured in order to evaluate the deterioration level of postmortem trout of each death time interval from biochemical and physical level. Histological analyses were performed in order to evaluate the deterioration level of the testes. Also, immunohistochemistry was carried out to testes by using double-immunofluorescence staining with two antibodies: anti-GFP antibody which can detect ASG of vasa-Gfp transgenic rainbow trout, together with anti-active caspase-3 antibody which can detect the apoptotic cells. To compare the deterioration level of testes in each group, the frequency of apoptotic germ cells, i.e. double positive GFP / caspase-3 cells, were quantified based on the immunohistochemistry results. Further, the germ cells were dissociated from the postmortem fish by enzymatic dissociation. The recovered germ cell numbers were counted in each group for the calculation of the total number of germ cells divided by net testicular weight in order to compare the efficiency of retrieved germ cells from postmortem fish. Next, to analyze their transplantability, 104 cells were transplanted into the body cavity of newly hatched larvae of rainbow trout. The transplantation efficiencies were evaluated by dissecting recipients and observing their gonads at 25, 90 and 150 days-post transplantation (dpt). As a result, ATP concentration were gradually decreasing in time-dependent manner and had a significant decreasing since 12 hour-post-death (hpd). At 24 hpd, the ATP was nearly undetectable. For rigor index, its value increased from 6 hpd to 12 hpd but significantly

decreased at 24 hpd. The results of biochemical and physical levels of postmortem fish indicated that the rigor mortis were almost completed around 12 hpd by exhaustion of ATP under the temperature of 10.5℃. According to the result of histology, the testicular cells lost normal morphology and nucleus shrinking occurred in ASG, especially when death time increased to 24 hpd. However, the germ cells with strong GFP-positive signals of green round-shape could be detected in all 0, 6, 12 and 24 hpd testes, while in 24 hpd, the antibody signals became weaker compared with the other groups. From the result of double-immunofluorescence staining, the apoptotic germ cells in the testis were found in all groups with an irregular shape. The frequency of apoptotic germ cells increased with the time-dependent manner and reached the highest at 12 hpd but decreased at 24 hpd. Statistical analysis proved no significant difference between them. Germ cells then dissociated from testes in each group showed the clear GFP signals. There was no significant difference in the total number of germ cells divided by net testicular weight in all groups, although cell number recovered from the 24-hpd group showed smaller tendency. As a result of germ cell transplantation, the transplanted ASG derived from each group had been found to incorporate into genital ridges of the recipients at 25 dpt. The transplantation efficiency of recipients which carried donor derived ASG were quantified. The transplantation-success rates at 25 dpt of 0, 6, 12 and 24 hpd testes were 86.29 ± 5.70%, 82.22 ± 11.76%, 73.33 ± 3.33%, 6.68 ± 6.66%, respectively. The incorporated GFP-positive-cell numbers of genital ridges from 0, 6, 12, and 24 hpd were 10.88 ± 4.00, 11.02 ± 3.80, 6.45 ± 1.91 and 1.17 ± 0.60, respectively. There were a significant difference between 24 hpd group and the others. Further, large cell clusters showing green fluorescence were observed in both testes and ovaries of recipients receiving germ cells isolated from 0-12 hpd fish at 90 dpt and 150 dpt. Both the proliferation and differentiation of the incorporated germ cells were observed in the female and male recipients of 90 and 150 dpt. However, in the recipients receiving the ASG retrieved from fish of 24 hpd group, only one specimen possessing GFP-positive oocytes in their gonads were observed at 90 dpt. These results suggested that ASG retrieved from fish of 24 hpd group barely contained viable or functional ASG, which can differentiate normally into female germ cells in the genital ridges of the recipients. In 90 dpt, the frequency of recipients which carried GFP-positive colonies of germ cells derived from fish of 0, 6, 12, and 24 hpd groups were 77.77 ± 14.70%, 77.77 ± 5.53%, 55.57 ± 5.57%, and 5.56 ± 5.56%, respectively. In 150 dpt, the frequency of recipients which carried GFP-positive colonies of germ cells derived from fish of 0, 6, 12, and 24 hpd groups were 75.70 ± 9.74%, 78.23 ± 13.71%, 53.9 ± 8.40%, and 0%, respectively. No significant difference of these values were found among 0, 6, 12 hpd at both 90 and 150 dpt. In conclusions, in this study, we successfully evaluated the freshness level of the postmortem fish by measuring ATP dosage and rigor index as indicators revealing the correlation between viability of germ cells and their transplantability. ASG retrieved from rainbow trout within 12 hours after their death,

are proven to maintain high viability and transplantability and to undergo successful differentiation of either oogenesis or spermatogenesis in the ovary and testis of recipient, respectively. Thus, germ cell transplantation system using postmortem fish developed in this study enables it to pave the way toward production of offspring derived from the dead fish. The germ cell transplantation can be a convenient emergency tool for the preservation of genetic resources even though postmortem fish.

Introduction

The increased demand for fish and the human activities have resulted in rapid depletion of fish stocks in the whole world. A number of fish species now therefore face extinction due to overfishing and habitat destruction (Shaffer et al., 1981; Meffe et al., 1997). Given these trends, there are urgent requirements to take action to preserve these endangered fish. Until now, considerable effects have already made for preservation, for example, to establish the close areas and season for fishing to repair fish habitat. Even more, the ex-site conservation like long-term captive breeding program for recovering the critically endangered species play a crucial role (Robert, 2009). Meanwhile, to satisfy human consumption, large effect has made to improve the efficiency and sustainability of the aquaculture industry. For example, researchers have worked on producing the new varieties that have desirable traits, such as rapid growth, high meat quality, increased fecundity, stress resistance and early spawning. In recent years, selective breeding has been successfully applied to improve the desirable traits in multiple species including rainbow trout (*Oncorhynchus mykiss*) (Kause et al., 2005), silver carp (*Hypophthalmichtchys molitrix*) (Gheyas et al., 2009), channel catfish (*Ictalurus punctutatus*) (Rezk et al., 2003), new Gift tilapia (GIFT, *Oreochromis niloticus*) (Li et al., 2010) and bastard halibut (*Pseudosciaen acrocea*) (Liu et al., 2005), large yellow croaker (*Pseudosciaen acrocea*) (Ning et al., 2007). These selected fish strains in general carry the valuable genetic resources for aquaculture.

Currently, a large number of aquaculture farm or research institution have been maintaining the endangered fish species or these valuable fish strain by captive breeding. However, captive breeding is typically accompanied with many limitations such as high economic costs, labor, potential risk like pathogen infection, or facility accidents like the

breakage of water supply, air pump, heater. If these kinds of accidents happen, all the fish in the same plot will die, especially for small population size. If so, nothing would be left and everything would be vanished including fish itself and all the efforts for fish breeding. Most importantly, the genetic information of endangered fish and commercially valuable fish will be also lost forever. This sudden loss of fish strain is irreversible which will take its huge toll since happen. Since the vulnerability of aquatic breeding system, although the issue can be addressed to take the necessary precaution, there is still risk causing fish death and loss of fish genetic resources that unable to store their strain in a short time, suggesting that emergency conservation strategies are required to provide a viable method for the conservation of these dead fish.

For those valuable species, whose cells and tissue may have been cryobanked before can recover their genetic resources conveniently anytime. However, when the event of such an emergency occurred to cause fish death there is no guarantee for cryobanking of all the species of fish in advance. In this case, for the valuable dead strain without cryobanking, how to preserve them should be emphasized in priority in case of losing their genetic resources. It was founded that the spermatozoa of dead fish remained viable for some time after death (Koteeswaran and Pandian, 2002). Since cryopreservation of spermatozoa has been well established in several fish species (Chao and Liao, 2001; Suquet et al., 2000; Babiak et al., 1997; Lahnsteiner, 2000), squeezing spermatozoa from the dead fish and perform cryopreservation for producing fish in appropriate time by insemination is a considerable method. However, spermatozoa may not be viable when the dead fish are found not in time. Especially when the postmortem storage was prolonged for more than 40-60 min, the sperm motility duration was lowered and sperm motility characteristics were significantly influenced in rainbow trout (Dietrich et al., 2005). Simultaneously, not all the spermatozoa from dead fish can be obtained any time, especially in non-spawning season or

from immature fish. Therefore, it is impractical to preserve the dead fish under different condition only by insemination method. Hence, the rescue strategies must be required for preserving these valuable genetic resources of postmortem fish for long-term and more practical applying when the disease outbreak or facility accidents happen.

In recent years, spermatogonial transplantation has been developed in various kinds of fish species, such as salmonids (Okutsu et al., 2006, 2007; Yoshizaki and Lee, 2018), Nile tilapia (Lacerda et al 2006), zebrafish (Nóbrega et al., 2010), yellowtail (Morita, et al., 2012), carp (Patra et al., 2016), medaka (Seki et al., 2016) and cyprinids (Octavera and Yoshizaki 2019). In this technique, singly germ cells dissociated from donor fish, are transplanted into body cavity of a newly hatched recipient larvae. Then, a small portion of type-A spermatogonia (ASG) contained in these transplanted donor cells migrate towards the genital ridge and be incorporated into them. Further, the incorporated type-A spermatogonia are differentiated into functional eggs and spermatozoa in female and male recipient, respectively. Finally, just by mating these two gametes of recipients, offspring carrying the donor derived genotype can be produced (Takeuchi et al., 2003; Kobayashi et al., 2007; Saito et al., 2008; Morita, et al., 2012; Seki et al., 2017; Hamasaki et al., 2017; Octavera and Yoshizaki, 2020; Nagasawa et al., 2019; Kawasaki et al., 2012). This technique is expected to be a promising application in the field of genetic preservation and aquaculture. It is a powerful alternative to makes it possible to inherit the valuable genetic resources of the fish to next generation and hence preserve the particular fish strain carrying desirable phenotypes and the endangered fish species (Yoshizaki et al. 2011).

In spermatogonial transplantation, viable germ cells isolated from just sacrificed donor fish which possess the transplantability are transplanted into the recipient where they can colonize the genital ridge and proliferate for self-renew and become numerous differentiated germ cell. Therefore, if the donor germ cells derived from postmortem fish are viable and

have function, once transplanted into the body cavity of recipient where a good body environment is provided, it is theoretically that they can also proliferate and different. The question is whether the germ cell dissociated from postmortem fish are still alive and whether the viable dissociated germ cells possess the function or transplantability once applying spermatogonia transplantation, since the postmortem fish have undergone some kinds of deterioration. Therefore, evaluation of the postmortem fish and exploring the feasibility for germ cell transplantation of these germ cells derived from these deteriorated postmortem fish is necessary for preserving the valuable dead fish by applying this technique.

Until now, evaluation of postmortem fish have been reported for some species, however, most such investigations have focused on the quality of seafood meat. There are no published data relating to the cell viability in the postmortem fish. Since the evaluation of the deterioration level of postmortem fish has been already revealed from its muscle level, which suggested that their indicators may be helpful for revealing the correlation of the evaluation of the deterioration level from cellular level with that feasibility for germ cell transplantation.

Therefore, to figure out whether postmortem fish inside of tanks or net cages which undergo deterioration possess the viability and function of germ cells could be preserved by germ cell transplantation, we performed four experiments to evaluate the deterioration level of postmortem fish and investigate the transplantability of germ cells derived from these postmortem fish. In this study, we measured the ATP concentration and rigor index as the freshness indicator. Testes were evaluated from cellular level and ASG retrieved from different levels of deteriorated postmortem trout and were transplanted into the recipient and their transplantability and differentiation ability were evaluated by applying germ cell transplantation technique.

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Experiment 1
Evaluation of the freshness level of postmortem fish

1.1 Introduction

If pathogen infection or facility accidents happen, the valuable fish face death immediately. In this case, deterioration had inevitably occurred to the fish when we find it. To preserve these valuable dead fish, we intent to perform the technique of germ cell transplantation with the detail steps which are to dissociate germ cells from these postmortem fish and transplant them into the body cavity of the recipient. Before we investigate whether the germ cell derived from these postmortem fish have the possibility to maintain their viability and transplantability, the priority is to evaluate the deterioration level of postmortem fish because what quality of the entity we got in hand is directly have an effect on the quality of germ cells and the efficiency of germ cell transplantation.

The investigations about the evaluation of the deterioration level have mainly focused on the postmortem muscle especially for the seafood. Even though the deterioration process itself is affected by a multiple of factors, the pathway to decompose after individual death is similar. Therefore, mastering the process of deterioration occurred to the dead body, especially to the well-studied muscle, is helpful for our understanding of the deterioration level of postmortem fish and for future exploration of the feasibility of germ cell transplantation.

In general, with the onset of death, due to the stop of heart beating and blood circulation, oxygen no further supply to the body. ATP (adenosine 5’- triphosphate) which is used as an

energy source by every living organism and found in every living cell, is still synthesized for a short of times by creatine phosphokinase through PCr (Phosphocreatine) reaction then by glycolysis after glycogenolysis (Needham and Carnis, 1971). The carbohydrate glycogen stored in the body is anaerobically degraded and lactic acid then accumulates in the muscle resulting the decrease of pH and a series of biochemical process (Nazir and Magar, 1963; Watabe et sl., 1989). The temporal pattern of the postmortem ATP dosage relies on the PCr and glycogen stock at the onset of death and the period of anoxia. When ATP content begins to decrease by enzyme reaction (Watabe et al., 1991) to a level, it initiates the crossbridge cycling between myosin and actin in myofibrils ceases, and actin and myosin linkages formed irreversibly resulting in the stiffness of the whole body and set in the rigor mortis (Iwamoto et al., 1987; Pate and Brokaw, 1980). The relationship between ATP and rigor mortis was first found by Erdo and many researchers confirmed that conclusion under different conditions (Erdo, 1943; Bendall, 1973; Hoet and Marks, 1926). On the other word, the onset and extent of rigor mortis are biochemically characterized by the depletion of ATP. Rigor mortis generally begin to appear 2-4 h after death and develops fully by 6-12 h (DiMaio and DiMaio, 1993). When ATP exhausted, the resolution of rigor mortis happen which make the muscle less rigid and no longer elastic. Therefore, with time pass by after death, these postmortem changes including the stiffness of muscle, ATP degradation correlate well with the loss of freshness of the dead body indicating that they can be used as the indices to evaluate the deterioration of fish.

Currently, a range of methods for the evaluation of corpse based on the changes of postmortem associated with sensory, biochemical and physical properties and microbiological growth have been reported (Gökodlu, Özden and Erkan, 1998; Huss, 1995).

In fish, the organoleptic methods, also organoleptic testing, is using human sense to examine the properties on the postmortem fish such as the appearance, odor, texture. They

can be applied to all fish species, require no facilities and are quick and non-destructive. There are several grading methods used to assess the freshness of fish such as the European Union scheme, the quality index method, the torry scorning system (Tahsin et al., 2017) for the quality assessment of raw fish in the inspection service and fishing industry. This method is rapid and suitable for overall judgment but without doubt is subjective, especially not specify the different species and varied conditions. Therefore, to quantify the result, the clear evaluation standards are required for varied types of fish under different conditions for the objectivity purpose. Also, sensory evaluation of the freshness should be conducted to make sure they are concurrently with the result of objective methods like chemical and physical methods (Alasalvar et al., 2001).

Compared with the sensory method, biochemical method is more objective to determine fish freshness and quality. In the muscle of postmortem fish, a variety of chemical compounds are accumulated. These chemicals are either intermediate or end products of biochemical changes occurring during in the deterioration of fish after death. Therefore, these changes during deterioration can be useful indices of the quality of postmortem fish. Among them, the most used compounds are volatile base nitrogen (TVB), ATP concentration or its decomposed products (Ryder, 1985), organic acids, nonprotein nitrogen, histamine, and indole and so on. Especially, the change of ATP concentration is faster than other changes (Uchiyama et al., 1970) and correlates well with the loss of freshness in a wide range of fish. These are evaluation generally dependent on instruments avoid subjecting to human variation and thus are more easily reproducible and reliable.

In the physical methods, measurements are mostly based on the changes of properties such as rigidity/rigor index (Bito et al., 1983), shear strength (Montero and Borderias, 1990), and isometric muscle strength (Nakayama et al., 1992). The rigor index which reflects the hardness of a fish body and the changes during deterioration are quantified by just measuring

the difference between the tails under different deterioration level via a vertical desk (Bito et al., 1983). This method was used to monitor the development of rigor mortis and the rigor mortis states of the fish were classified according to the following criteria: (1) pre-rigor mortis: no stiffening, full movement of muscle (rigor index ≤ 10%); (2) in- rigor mortis: fully stiffened (80% ≤ rigor index ≤ 100%); (3) post-rigor mortis: the state when rigor index ≤ 10%. The in-rigor mortis period contained in the onset period, which was from the first detectable stiffening through full development of stiffening. The onset period was indicated by 10% < rigor index ≤ 100% (Wang, et al., 1998).

The microbiological methods were established based on the fact that the bacteria from skin, gills and intestines may proliferate freely since fish die. The evaluation was performed by using microscopy, and specific metabolic evaluation (TMA) for the numbers (Gibson et al., 1997) and immunology or molecular biology for identifying the pathogenic bacteria (Ogden et al., 1997).

It has been commonly considered that no single method is reliable for assessing the freshness and quality of fish. Therefore, a combined methods have been recommended for the evaluation of freshness and quality. Essentially, one objective of this research is to investigate the deterioration level of postmortem fish in an attempt to correlate that result with the feasibility for germ cell transplantation. To fulfil this purpose, choosing the objective and convenient methods for evaluation of the postmortem fish is essential since any misjudgment or prolonged complex procedure will cause losses, miss the appropriate time for preserve, make the dead fish deteriorate further. Therefore, to evaluate the deterioration of postmortem fish, in this study, we decided to combine two methods of physical and biochemical to measure the ATP concentration and rigor index which are closely related to time lapse as the indicators of the deterioration level of postmortem fish.

In this experiment, the objectives were: (1) to measure the rigor index and ATP

concentration of muscle in postmortem rainbow trout at 10.5℃ flowing water for evaluation of the deterioration level; (2) to examine these two indies are corresponding to the process of deterioration of postmortem trout under experimental condition. Meanwhile, postmortem fish which die for as long as 24 hours will be our experimental fish.

1.2 Materials and Methods

1.2.1 Ethics

All experiments were performed in according with the Guidelines for the Care and Use of Laboratory Animals of the Tokyo University of Marine Science and Technology, Tokyo, Japan.

1.2.2 Fish

Nine-month-old male *vasa-gfp* rainbow trout whose ASGs are specifically labeled with green fluorescence protein (GFP), were used in the present study. All fish were maintained at the Oizumi Station of Field Center of Tokyo University of Marine Science and Technology (Yamanashi, Japan) under a 7 hours (h) light and 17 h dark photoperiod at a water temperature of 10.5℃ and fed three times per day.

1.2.3 Preparation of postmortem fish

All rainbow trout were allocated randomly to four groups and killed by anesthetization with high concentration of 2-phenoxyethanol (Wako, Osaka, Japan). Then the dead fish were incubated in 200-l fiber-reinforced plastic tanks with flowing rearing water at 10.5℃ for 0, 6, 12 and 24 h, respectively. Total length (TL, cm), body weight (BW, g) and gonadal somatic index (GSI, %) of postmortem fish were measured before incubation. Three repetitions are performed for each group.

1.2.4 Evaluation of ATP dosage of dead fish

In order to evaluate freshness of each incubated group, ATP concentration of muscle tissue and rigor index of whole fish body were calculated. Approximately 20 mg of muscle

tissue were collected from postmortem fish and then were lysed and processed according to the manufacturer's instructions of a colorimetric ATP assay kit (ab83355, Abcam, England). The ATP dosage was calculated using a microplate absorbance reader at a wavelength of 570 nm (Bio-Rad 680, USA).

1.2.5 Evaluation of rigor index of dead fish

To evaluate the stiffness of postmortem fish, rigor index is calculated which reflect the stiffness in quantity. Rigor index was calculated individually by using horizontal displacement method (Iwamoto et al., 1987). Half body length of fish body was removed to the edge of the table and the changes of vertical drop (cm) of the fish tail were recorded. Rigor index (Ir) was calculated according to the formula adapted from Bito et al (1983), modified by Masette and Kasiga (2007). $Ir = [(L0 - Lt) / L0] \times 100$, where L0 is vertical distance from the top of the horizontal table edge to the fish tail fork at time of death, while Lt represents the same measurement at postmortem sampling times.

1.2.6 Statistical analysis

All data were described as the means ± standard error of the mean (SEM). Statistical analyses were performed using SPSS software (Version 17.0 for Windows). Differences were analyzed by using a one-way analysis of variance and Tukey-Kramer's multiple comparisons test. P values < 0.05 were considered as significant difference.

1.3 Results

1.3.1 The growth data of experimental fish

The fish data were collected from *vasa-gfp* rainbow trout incubated for 0, 6, 12, and 24 h under 10.5℃. The TL of 0, 6, 12 and 24 hpd were12.47 ± 0.45 cm, 12.58 ± 0.22 cm, 12.14 ± 0.14 cm, 12.29 ± 0.17 cm, respectively. The BW of 0, 6, 12 and 24hpd were 21.92 ± 2.01 g, 22.03 ± 0.94 g, 19.65 ± 0.84 g, 22.17 ± 1.07 g, respectively. The gonadal somatic index (GSI) of 0, 6, 12 and 24 hpd were 0.070 ± 0.004%, 0.067 ± 0.008%, 0.081 ± 0.005%, 0.067 ± 0.005%, respectively. All fish data have no significant difference between each group (Fig. 1.1).

1.3.2 Comparison of quality of dead fish by ATP dosage

To evaluate the freshness level of postmortem fish incubated in each death time, ATP dosage remained in the muscle tissue were calculated. As a result of ATP concentration, there was the tendency that ATP concentration were gradually decreasing in time-dependent manner. In 24 hpd, ATP dosage were not completely detectable. There was no significant difference between the ATP concentration of 0 hpd and that of 6 hpd (Fig. 1.2). However, a significantly decreasing was observed since 12 hpd.

1.3.3 Comparison of quality of dead fish by rigor index

To evaluate the freshness level of postmortem fish incubated in each death time, rigor index related to the stiffness of body were calculated. As a result of rigor index, its value increased with the lapse of incubation time from 6 hpd to 12 hpd. However, it had started to decrease at 24 hpd (Fig. 1.3). These results indicated that the rigor mortis was almost finished around 12 dph with nearly exhaustion of ATP under the temperature of

10.5℃.

1.4 Discussion

In this experiment, we evaluate the deterioration level of postmortem rainbow trout incubated in rearing water of 10.5℃ for 0, 6, 12 and 24 hours. As a result, ATP dosage decreased significantly from 6.74 to 0.02 μmol/g nearly totally exhausted at 12 hpd, at the same time, the rigor index reached the peak value compared to the other groups but had a decrease at 24 hpd. The results of ATP and rigor index indicated that the trout after death was experiencing shifting from the state of pre-rigor mortis to in-rigor mortis and to post-rigor mortis and there was high possibility that the onset of rigor mortis started around 6 hpd and the resolution of rigor mortis started around 12 hpd. When death time continued increase to 24 hpd, the rigor mortis was finishing with the decrease of rigor index value and almost exhaustion of ATP dosage. Therefore, measuring the chemical indices of ATP concentration and physical indices of rigor index proved their effectiveness in assessing the deterioration changes of the postmortem trout in this study. Based on the results from this experiment, the deterioration process of postmortem trout is not contradicted with what we aforementioned about the process of deterioration theoretically. Since ATP concentration and rigor index both with respect to the death time, thus they can be used as the good indicators for monitoring the loss of the muscle freshness whilst kept in tank since death.

As we have known that the onset of rigor in skeletal muscle of animals and fish is associated with consumption of ATP from muscle after death (Erdos 1943; Bate-Smith and Bendall 1949; Lawrie 1953; Partmann 1965). However, various factors such as exhaustion, starvation related to how fish die influenced the speed of the ATP metabolic and the progress of the rigor (Forster, 1963; Bate-Smith and Bendall, 1949; Krompecher and Frye, 1978; Ota et al., 1973; Krompecher and Bergerioux, 1988). In general, the onset of rigor mortis is hastened in fish due to exhaustion after struggling prior to death (Amano et al., 1953; Tomlinson et al., 1961). The initial remaining of glycogen or ATP since death were related

to the speed of rigor mortis development. Until now, the exact relationship between the rate and extent of the biochemical events in muscle and how environment factors affect these postmortem changes have not been clearly illustrated (Jerret et al., 1998; Sigholt et al., 1997; Proctor and Mcloughlin, 1992).

In the study, we killed the fish by using high concentration of phenoxyethanol in the quick method in which struggling at death does not occur. In this case, the experimental fish all had the similar level of energy stock when they died. Therefore, it avoided the interference from individual difference by stopping fish struggling. However, when fish die in the field by accident, they are exposed to asphyxiate condition and inevitable face struggling which therefore initiate a faster rate of ATP hydrolysis with the consequence of the speed up of anaerobic glycolysis and the advance of the onset of rigor (Proctor et al., 1992; Sebastio et al., 1996). That means, the deterioration of postmortem fish in the wild is not totally coincided with that of the fish in the experimental condition even under the same time interval after death. Therefore, the conclusion that the onset of rigor mortis started around 6 hpd and last around 12 hpd obtained by using the indices ATP and rigor index in this experimental condition are not suitable for other condition let alone other species. However, the process of deterioration of other fish species or other condition without doubt can be well indicated by using the ATP and rigor index as the indicators.

Despite we have already revealed the deterioration process of dead trout and could initially judge the deteriorated phase of dead fish by measuring the ATP concentration and rigor index, whether these dead fish are applicable to be used as donor for germ cell transplantation are still uncertain. Because it is the germ cells derived from gonad of these deteriorated fish that play an important role in deciding the successful transplantation. Since no reference introduced the deterioration of fish gonad, therefore, it is necessary to explore the deterioration of gonadal tissue, especially from cellular level, which also contribute to

the final assessing the feasibility for germ cell transplantation.

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1.6 Figure Captions

Fig. 1.1. The average of total length (TL, cm), body weight (BW, g) and gonadal somatic index (GSI, %) of 9-month-old male trout. Values are described as means ± standard error of the mean (SEM) and bars indicate SEM. Different letters indicate significant difference at $P < 0.05$ (Turkey-Kremer test).

Fig. 1.2: Evaluation of the freshness level of the post-mortem fish by ATP dosage. The ATP dosage contained in the muscle of dead fish for 0, 6, 12 and 24hpd fish. Values are described as means ± standard error of the mean (SEM) and bars indicate SEM. Different letters indicate significant difference at $P < 0.05$ (Turkey-Kremer test).

Fig. 1.3. Evaluation of the freshness level of the post-mortem fish by rigor index. The rigor mortis index (Ir) of 0, 6, 12 and 24hpd fish. Values are described as means ± standard error of the mean (SEM) and bars indicate SEM. Different letters indicate significant difference at $P < 0.05$ (Turkey-Kremer test).

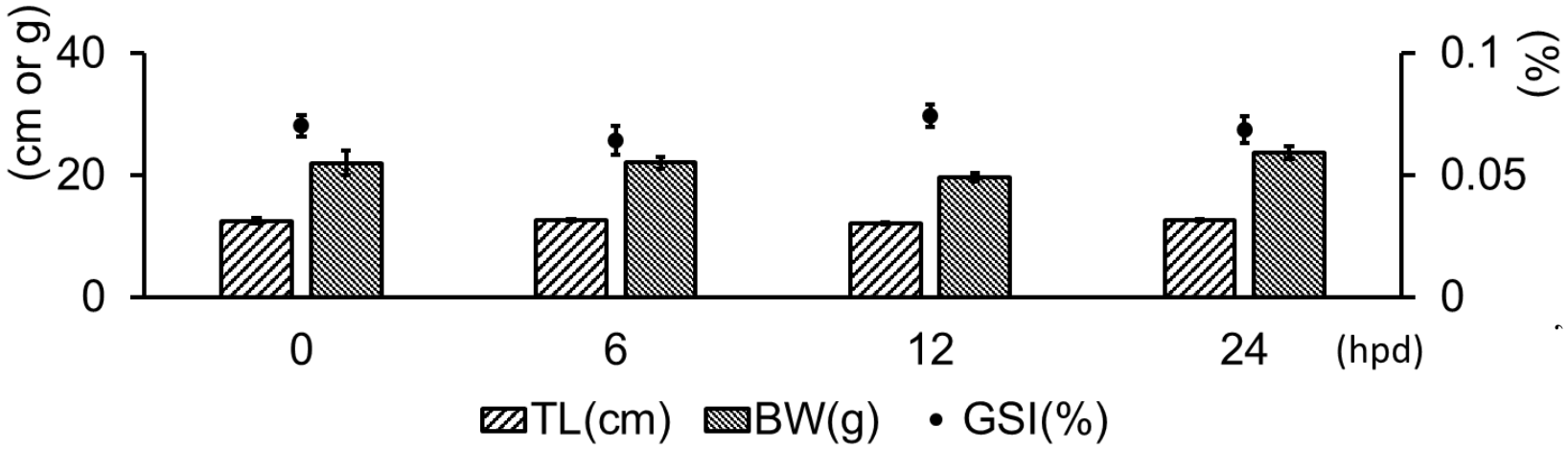

Fig 1.1 The average of total length (TL, cm), body weight (BW, g) and gonadal somatic index (GSI, %) of 9-month-old male trout

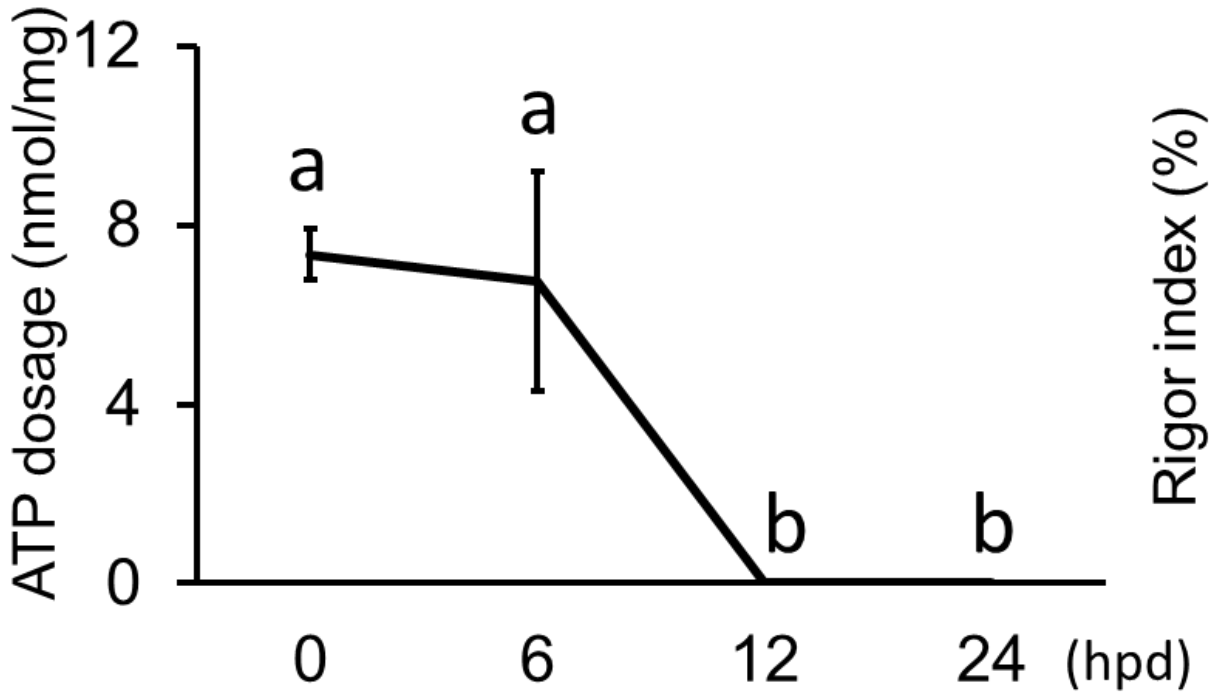

Fig 1.2 Evaluation of the freshness level of the postmortem fish by ATP dosage

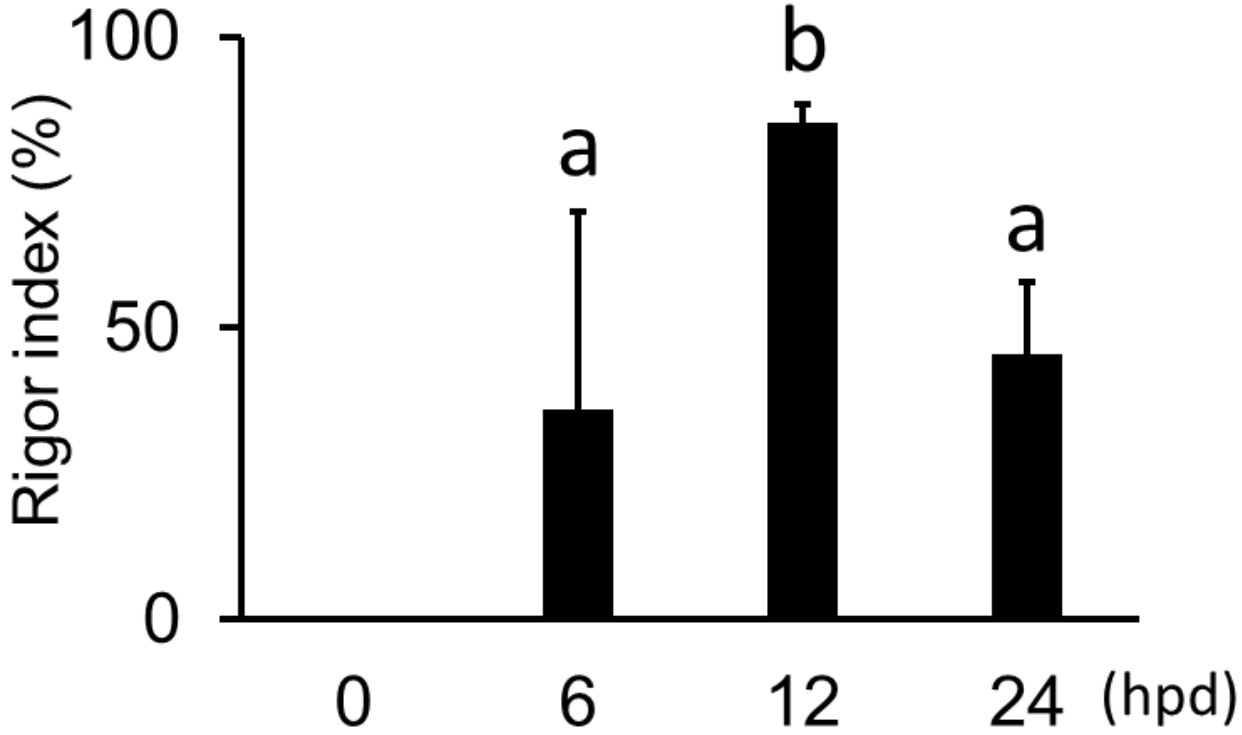

Fig 1.3 Evaluation of the freshness level of the postmortem fish by ATP dosage

Experiment 2

Evaluation of testes isolated from postmortem fish

2.1 Introduction

In order to preserve the postmortem fish by using germ cell transplantation, we firstly evaluated the deteriorated postmortem fish by measuring the indicators of ATP concentration and rigor index. However, the evaluation of postmortem fish is not enough to support predicting the feasibility of germ cell transplantability. To better correlate the feasibility of germ cells transplantation with the deterioration level of the dead fish, therefore, we have to evaluate the freshness of postmortem fish from tissue level and cellular level to have a deep understanding of the deterioration of the dead body inside.

It deserves to know that most tissues also respond in a similar way to the death signal. When fish is considered dead, the supply of oxygen and nutrition to cells are stopped, as well as the removal of metabolic wastes from cells, which are the result of the failure of respiratory and blood-pump by the heart. Stress like hypoxia and ischemia therefore occurred leading to each cell the imbalance of homeostasis. Even worse, intracellular ATP energy has the rapid depletion caused by the switch to anaerobic glycolysis. It is in fact just because the anaerobic generation of ATP that contributes to the short-term survival of the cells in vivo after individual death. Nevertheless, the resultant continuous deficiency of ATP leads to the failure of Na^+/K^+-ATPase pumps that control the cell volume and electrolyte balance with the eventually consequence of the influx of Na^+, Ca^{2+} and water into the cytosol and release of K^+ and Mg^{2+} from the cytosol. All of these are specifically manifested as acute cell

swelling. If possible that the stress circumstance can be relieved and imbalance of homeostasis only last for a short duration, cells in vivo may have the possibility to be recovered and returned to the normal structure and function. Therefore, the acute cell swelling in the dead body to some degree is the reversible process. That means, if we can retrieve germ cells which have reversible injury from postmortem fish and provide a suitable environment, the germ cells have high possibility to be rescued and even recover their function. This scenario offers us the chance to realize the preservation of the valuable dead fish by performing germ cell transplantation system.

However, if we spotted the postmortem fish not in time, initial acute cell swelling would become the first step towards the progress to irreversible cell death when the injury to cells is severe, even though it is not so severe as to be lethal injury that may not cause cell to succumb but is also unlikely to recover completely back to its initially structural and functional state when we provide a well environment. It is important to figure out that death is an end point but dying is a process that can be reversible until a first irreversible step is trespassed. What we expect is there are still large number of viable or dying germ cells with reversible injury from postmortem fish that can gain recovered after our dissociation. Therefore, exploring how cells die in vivo and distinguishing the death and dying cells are indispensable steps for the evaluation of deteriorated tissue and cells.

There are two distinct pathways about cell death based on biochemical and morphological characteristics, namely apoptosis and necrosis (Majno and Joris, 1995, 2004; Martin et al., 1998; Northington et al., 2001). Apoptosis, or called programmed cell death, is tightly regulated by molecular mechanisms and highly evolutionary conserved in the organism and play an important role in removing the unnecessary cells (Steller, 1995). During apoptosis, the plasma membrane fragments separate from the dying cells, which forms apoptotic body. The apoptotic cells lose contact with their neighbors and are

characterized by cellular volume reduction (pyknosis), nucleus fragmentation (karyorhexis), plasma membrane blebbing, maintenance of an intact plasma membrane and chromatin condensation until the late stage of this process (Green, 2011; Kerr et al., 1972; Wyllie et al., 1980). The major mechanism of apoptosis can be classified as extrinsic and intrinsic in which death receptors and caspases or mitochondria play a crucial role, respectively. For intrinsic pathway of cell death, it is triggered by a variety of stress-inducing stimuli, such as hypoxia, DNA damage that converge on the mitochondria. Mitochondria are organelles where the ATP are efficiently generated in part with cell metabolism. Meanwhile, mitochondria play an important role in cell death which induce apoptosis through activating the related caspase protein when the integrity of the mitochondrial membrane is ruptured (Tait and Green 2010; Liu et al., 1996). By contrast, cellular necrosis is considered to be a passive degenerative progress that results from cellular metabolic collapse and characterized by electron-lucent cytoplasm, cellular swelling, loss of plasma membrane integrity, dilation of cytoplasmic organelles, nuclear distension, moderate chromatin condensation (Green, 2011; Nikoletopoulou et al., 2013; Majno and Joris, 1995; Hawkins et al., 1972; Alison and Sarraf, 1994). Unlike apoptosis, necrosis is not associated with a genetic program and occurs independently of caspase activation (Leist and Jaattela, 2001). Apoptotic cells that are not efficiently cleared by phagocytes in vivo can undergo secondary necrosis.

Meanwhile, according to the suggestion of Nomenclature Committee on Cell Death (NCCD) that a cell can be defined as dead cells when any of the following criteria are met: (1) the cell has lost the plasma membrane integrity; (2) the nucleus of cells has undergone complete fragmentation into discrete bodies; (3) the corpse of cells have been engulfed by an adjacent cell in vivo. Therefore, cell death can be judged according to its morphological appearance (such as apoptotic or necrotic), enzymological criteria, functional aspects or immunological characteristics. Worth mentioning, necrotic cells which are featured by the

rupture of cell membrane based on NCCD is to some degree judged as dead cells.

Therefore, to evaluate the deterioration of postmortem fish from cellular level, it is better to find the bridge or turning point between apoptotic cells and necrotic cells. In general, there are two most common methods available to identify cell death at a single time point. One is routine histological stains, hematoxylin and eosin (H&E) which can be quickly and easily performed on tissue sections for the investigation of morphology features of postmortem testes. Histology enables to visualize dead or dying cells from tissue level. Apart from histology, another is biochemical methods like immunohistochemistry by using fluorescence markers of cell death for evaluation and quantification of cellular damage or death in experimental condition. Since the massive caspase activation may be one of the prerequisites toward apoptosis, which make it possible to be a prediction of death realizing through detecting of active caspase. Among the cascades of caspase protein, caspase-3 is the primary executioner caspase in apoptosis, required for the mass proteolysis that leads to apoptosis. Thus, the antibody to detect caspase-3 activity seems to be appropriate to use in tissue sections by performing immunohistochemistry (IHC) method.

In this experiment, in order to evaluate the deterioration of testes derived from postmortem fish, we performed evaluation based on histology and immunohistochemistry method for the testes derived from postmortem fish aim to compare the cellular changes of each group.

2.2 Materials and Methods

2.2.1 Immunohistochemistry analyses

To make testicular section, testes from dead fish incubated for 0, 6, 12 and 24 hours were fixed in Bouin's solution at 4°C for 12 h, embedded in paraffin wax, and then sliced into 4-μm-thick sections. Paraffin sections were dewaxed and rehydrated through a xylene-ethanol series. Antigen retrieved treatment were conducted against the rehydrated sections by using Histo VT One solution (Nacalai Tesque, Kyoto, Japan) at 90°C for 20 min. After treatment, in order to decrease non-specific antibody bindings, the sections were blocked by Block-Ace (DS Farmer Biomedical, Osaka, Japan) at room temperature for 30 min. After blocking, the sections were incubated for 16 h at 4°C with a primary antibody. Mouse anti-GFP antibody (11 814 460 001; Roche, Basel, Switzerland) and rabbit anti-caspase antibody (ab4051, Abcam, England) were used as primarily antibodies. The dilution rate of anti-GFP antibody and anti-caspase-3 antibody were both 1:1000 with Can Get Signal A (Toyobo Co., Osaka, Japan). After the immunoreactions, sections were washed three times for 5 min each with Phosphate Buffered Saline (PBS) containing 0.1% Tween 20 (PBST; Merck KGaA). After washing, the sections were incubated together with secondary antibody goat anti-mouse IgG and anti-rabbit IgG conjugated Alexa Fluor 488 and 546, respectively (Invitrogen Corporation). The secondary antibodies were both diluted to 1:200 with Can Get Signal B (Toyobo). After the immunoreactions, the sections were washed three times by PBST and then observed under fluorescent microscopes (BX-223 51; Olympus, Tokyo, Japan).

2.2.2 Histological analyses

To evaluate the quality of testes isolated from dead fish, histological analyses were

performed following the IHC. The slides were counterstained staining with hematoxylin and eosin (HE). After staining, the slides were dehydrated through an ethanol-xylene series and mounted using Entellan New (Merck KGaA). All sections were photographed using a fluorescent microscope (BX-223 51) with a digital camera (DP-70; Olympus).

2.2.3 Comparation of the ratio of apoptotic germ cells

To evaluate the deterioration level of incubated testes, the ratio of apoptotic germ cells were calculated in each group based on the immunohistochemistry results. The total number of apoptotic germ cells: both caspase-3 and GFP-positive cells were counted. The ratio of apoptotic germ cells is calculated by using the total number of apoptotic germ cells to divide the total number of germ cell: GFP-positive cells.

2.2.4 Statistical analysis

All data were described as the means ± standard error of the mean (SEM). Statistical analyses were performed using SPSS software (Version 17.0 for Windows). Differences were analyzed by using a one-way analysis of variance and Tukey-Kramer's multiple comparisons test. P values < 0.05 were considered as significant difference.

2.3 Results

2.3.1 Evaluation of testis quality of dead fish by immunohistochemistry

In order to examine the quality of testes isolated from postmortem fish, immunohistochemistry was carried out using double-immunofluorescence staining with two antibodies: anti-GFP antibody which detect ASGs of *vasa-Gfp* transgenic rainbow trout, together with anti-active caspase-3 which detect the apoptotic cells. As a result, the strong GFP-positive signals of green round-shape can be detected in all 0, 6, 12 and 24 hpd testes, while in 24 hpd, the antibody signals became weaker compared with the other groups (Fig. 2.1). While both GFP and caspase-3 double-positive cells indicating apoptotic germ cells in the testis were found in all groups with an irregular shape.

2.3.2 Evaluation of morphology of testis of dead fish by histological analyses

HE staining were performed following immunohistochemistry to observe the morphology of germ cells in the same specimens (Fig. 2.1). In non-incubated testes, a sparsely fragmented nucleolus and clear cytoplasm can be observed in germ cells. However, when time lapse, the testis tissue turned to become loosen and germ cells had a bit swell, especially in the12 hpd. On the contrary, in 24 hpd, the morphology of germ cells shrank a lot and becoming small round shape by histological observation.

2.3.3 Counting the frequency of apoptotic germ cells of the testes

To evaluate the deterioration level of incubated testes, the ratio of apoptotic germ cells were calculated in each group. We counted the total number of apoptotic germ cells: both caspase-3 and GFP-positive cells which were shown in Fig. 2.2. As a result, the ratio

of apoptotic germ cells increased with the time-dependent manner and reached the highest at 12 hpd. Statistically significant difference of apoptotic germ cells ratio was only found between 12 hpd and 0 hpd. Although, the ratio of 12 hpd was higher than that of 24 hpd testes, there was no significant difference between them.

2.4 Discussion

In the present experiment, we evaluated the deterioration level of postmortem testes from cellular level. As a result, the nucleus of germ cells condensed in a time dependent manner. The morphology of germ cells changed dramatically when death time increase to 24 hours. The percent of active caspase-3 positive germ cells increased gradually from 0 hour and reached the highest at 12 hours but decrease significantly at 24 hours. These results that germ cells derived from postmortem trout as long as 12 hours post death were still caspase-3 active and maintained the basically morphological shape indicated that they may be apoptotic cells which have intact cell membrane.

As we aforementioned that apoptosis and necrosis are the two forms of cell death. Compared to necrotic cells, apoptotic cells have intact membrane which have possibility to be rescued from death. However, the typical characteristics of apoptosis are cellular volume shrink, nucleus fragmentation, plasma membrane blebbing, chromatin condensation which is proven from electron microscope. These phenomena were not clear by our histological result with HE staining. On the contrary, the volume of cells from 6, 12 hpd testes had a bit swell with the result of the precent of active caspase-3 positive germ cell increased which is the perquisite of apoptotic morphology, compared to 0 hpd. Therefore, the morphology of germ cells in the testes seems to be not compatible with the corresponding cell death pathway and it is difficult to distinguish the type of cell death just by simply histological result. In fact, when fish die, in this circumstance, it is difficult to consider cell death as either “programmed” or “accidental” since there are varied types of stimuli including hypoxia, ischemia, low intracellular ATP. Cell death is a complex process which involve many mechanisms. Until now, no researcher can give a clear answer for the exact cell death pathway after individual die since there are different cell types with varied reparative or

regenerative capacities. Studies indicated that postmortem muscle cells die through apoptosis (Becila et al., 2010). However, studies on the myocardial ischemia suggesting that the mechanism of cell injury and death in early stage involves both acute cell swelling (oncosis) and apoptosis in approximately equal degree (Buja and Vela, 2008; Buja and Weerasinghe, 2010; Foo et al., 2005).

Cell swelling, also called hydropic degeneration, have different names in different organ systems (e.g., cytotoxic edema in the central nervous system and ballooning degeneration in the epidermis) and is the most common and fundamental expression of cell injury. A lot of stimuli which cause the loss of the cell's homeostasis secondary to mechanical such as hypoxic, toxic, free radical, viral, bacterial, and immune-mediated injuries can cause cell swelling. It is manifested as increased cell volume with an overload of water which were caused by the deficiency of ATP giving rise to the failure of Na^+-K^+ pumps and hence lead to the failure of the cell to maintain normal homeostasis. The swelling is accompanied by modification and degeneration of organelles, which is reversible cell injury process. Thus, the germ cells with acute cell swelling derived from postmortem fish may have the potential to be rescued.

However, worth mentioning, no matter acute cell swelling or apoptosis, they are commonly cell reaction facing to the change of intracellular ATP. It had been shown that the morphological change of apoptosis like chromatin condensation and nuclear fragmentation is ATP required (Kass et al., 1996; Leist et al., 1997). Although the role of ATP in apoptosis remains controversial, low level of ATP can contribute to cell death by necrosis which have a disruption of plasma membrane (Leist et al., 1997; Decaudin et al., 1998). Therefore, in conclusion, the decrease of ATP is the signal for cell death. As we mentioned before, when ATP exhausted, the myofilaments are unable to slide over each other, and thus resulting in rigor mortis which infact occurs on an individual cell level but acts over the whole fish,

making the postmortem fish stiff as rigor develops. According to Erikson (1997), compared to the stressed fish, the comparatively “rested” fish still contain ATP even when the fish reach the peak rigor indicating that some cells had not entered rigor even though the majority of cells had resulted in the fish become stiff during the rigor process. Even though, we performed ATP measurement of the muscle to evaluate the deterioration of the whole fish in Exp. 1, the difference of metabolites in cells in tissue seem to be able to indicate that the result of intracellular ATP concentration in muscle used for the indicator of postmortem fish is not suitable for testes tissue. Although the decrease of ATP is the signal for cell death, the indicator of ATP concentration cannot totally satisfy the purpose to evaluate the quality of germ cells in testes because we aim to achieve the viable germ cell from postmortem fish and use for germ cell transplantation.

In apoptotic cells, due to the reduction of ATP, the permeability transition pores open. The mitochondrial membrane integrity has been reported to be therefore disrupted which result in the release of apoptogenic factors into the cytosol. Cytochrome C together with apoptosis-inducing factor (AIF) finally complete the cascade and activate the caspase-3 protein and other endonucleases (Liu et al., 1996, 1997; Zou et al., 1997). The main function of caspases is to cleave proteins to make sure efficient and rapid cell death. Structural components such as nuclear lamins and cytoskeletal proteins that bind to the plasma membrane are cleaved by caspases, then the nuclear condensation and membrane blebbing occur (Ctyns and Yuan, 1998). Therefore, caspase-3 is as it is the effector caspase to activate the cell death signaling in the nucleus (Samali et al., 1999). Some evidence showed that a shift in morphology from apoptosis to necrosis occur when there is inhibition of caspases (Lemaire et al., 1998) which related to a direct loss of energy metabolism or osmotic stability (Leist et al., 1997). Therefore, active caspase-3 is undoubtedly the suitable indicator for monitoring the deterioration of germ cells in the testes. In this experiment, we

use active caspase-3 antibody in immunohistochemistry and measured the percentage of active caspase-3 in different postmortem testes. The result showed that the ratio reached the highest value at 12 hpd while decrease significantly at 24 hpd. The frequency of caspase-3 activated germ cells decreased at 24 hpd fish seemed to indicate the shifting from apoptotic cells to further deteriorated germ cells like necrotic cells. Based on these results, we got conclusion that most of the germ cell in the testes which experienced some acute swelling histologically accompanied by the increased caspase-3 activation may entered into apoptosis pathway gradually until 12 hpd. However, since no energy and no oxygen supplemented to the cells in the postmortem fish and the imbalance homeostasis got further worsen, the germ cells with decreased activated caspase protein out of being inhibition or other reason at 24 hpd may be shifted to undergo necrosis.

However, based on the result from the indicators of morphology and the percent of activate caspase-3 antibody, it is not enough to prove that the apoptotic cells have ability to be recovered and still be viable. Whether germ cells from the postmortem testes are still in the reversible apoptosis stage and still viable is unknown, as well as their function like transplantability in germ cell transplantation system. Anyway, the morphology and the percentage of active caspase-3 of germ cells can be used as indicators since they reflected the deteriorated degree of testes under different dead time, which would help us to find the correlation with their feasibility for germ cell transplantation in the future.

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2.6 Figure Captions

Fig 2.1 Evaluation of the testis quality in postmortem *vasa-Gfp* transgenic rainbow trout. Immunohistochemistry with anti-GFP antibody, anti-caspase-3 antibody and HE staining of the testes of postmortem fish. Arrowheads indicate apoptotic germ cells which are GFP / caspase-3 double positive cells. Scale bars indicate 20 μm in all panels.

Fig 2.2 Frequency of apoptotic germ cells. Values are described as means (N=3) ± SEM and bars indicate SEM. Different letters indicate significant difference at $P < 0.05$ (Turkey-Kremer test).

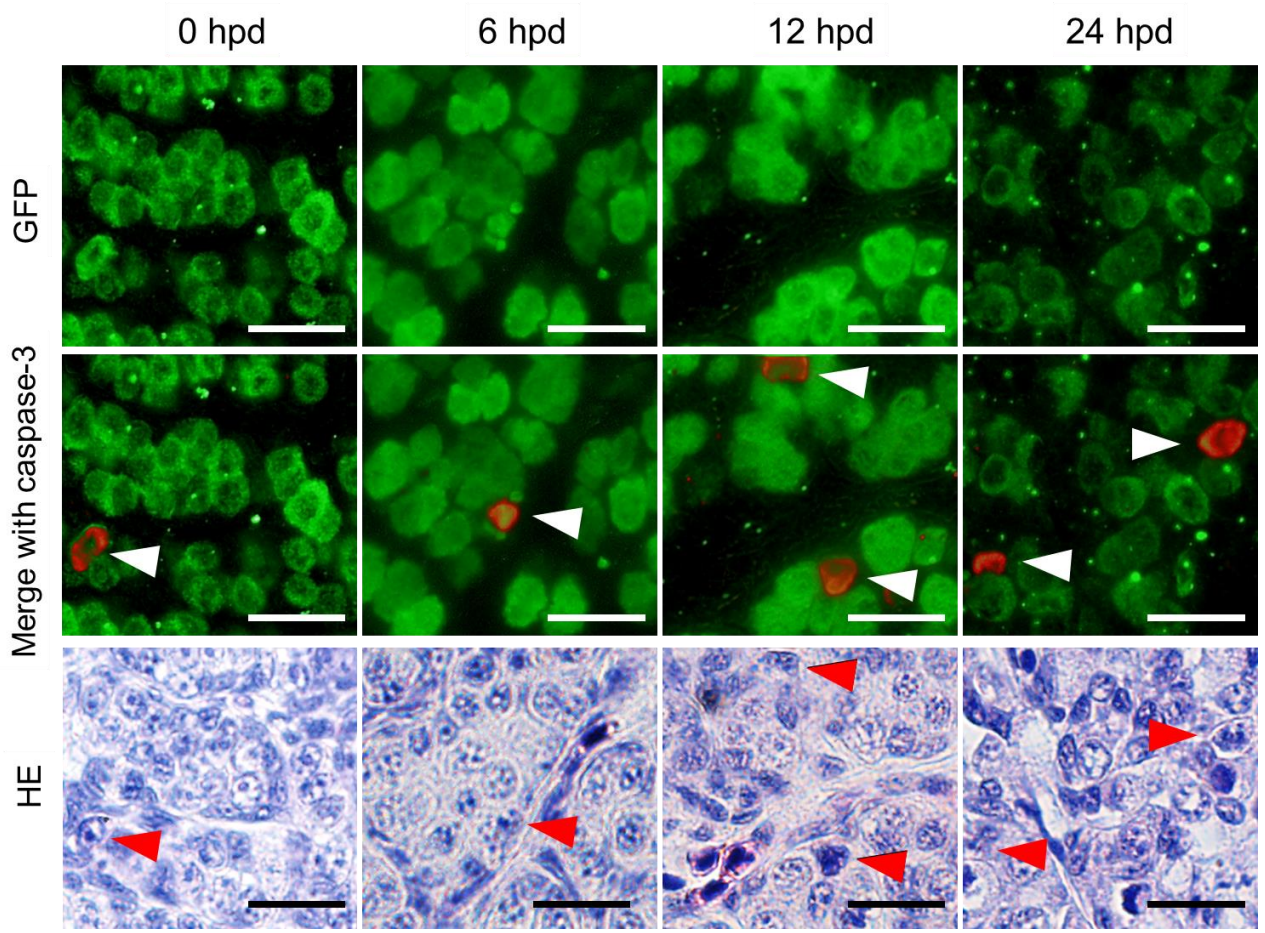

Fig 2.1 Evaluation of the testis quality in postmortem *vasa-Gfp* transgenic rainbow trout by immunohistochemistry with anti-GFP antibody, anti-caspase-3 antibody and HE staining of the testes of postmortem fish.

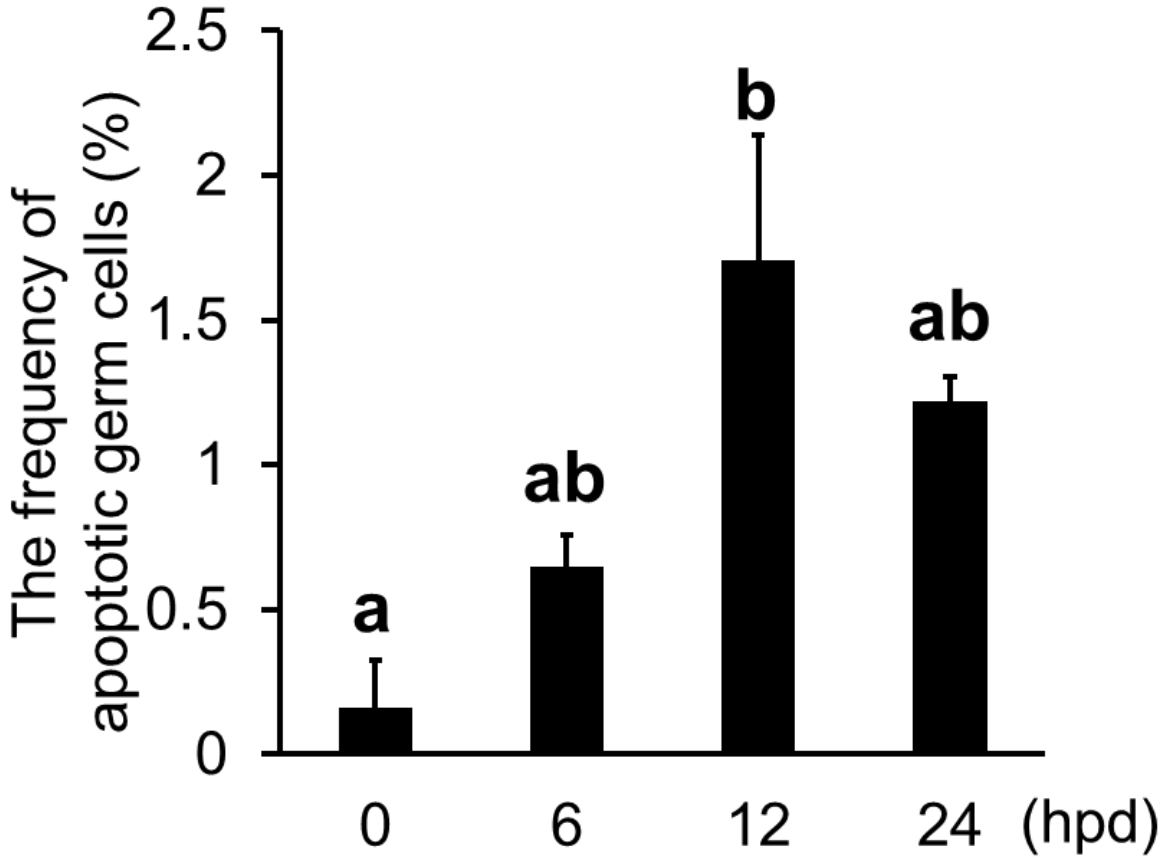

Fig 2.2 Evaluation of the testis quality in postmortem *vasa-Gfp* transgenic rainbow trout by frequency of apoptotic germ cells.

Experiment 3
Counting germ cell numbers retrieved from postmortem fish

3.1 Introduction

Since we have roughly evaluated the deterioration of fish and the deterioration of testes, however, our final goal is to obtain the transplantable germ cell from postmortem fish of which not only maintain the function but also should have a considerable population. As the levels of ATP in the cell population decreased, the percentage of viable cells also decreased. In the previous experiment, germ cells expressing active caspase-3 indicated that they were apoptotic germ cells which may still possess the intact plasma membrane. To prove whether we can obtain germ cells with intact plasma membrane, in next step, we dissociated germ cells derived from the postmortem testes. The evaluation of these germ cells would be performed in this experiment.

In our lab, we have developed a procedure to isolate whole germ cells from fish testes tissue (Okutsu). The technique utilizes enzymatic digestion with collagenase and trypsin to digest testes tissue to single cells after removing the attached unnecessary blood vessel. Thereinto, the collagenase can digest the collagen which is the backbone of the extracellular matrix that holds all cells in the tissue; the trypsin is a serine protease which non-specifically cleaves peptides on the c-terminal side of lysine and arginine amino aside residues or dissociate adherent cells and has been described for a variety of uses relating to dissociation of various tissue types. Mild pipetting is performed for the suspension acted as mechanical

dissociation in order to well isolate single cells. This technique has already applied successfully to varied fish species to yield cells from tissue.

Maintenance of cellular integrity after dissociation is one of the considerable important factors to judge the viable cells during evaluation. The principal concern of cell dissociation is that the proteolytic enzymes may penetrate the plasma membrane and damage the fragile cells which further increase the deteriorated level. Therefore, to evaluate the germ cells derived from postmortem testes, it is necessary to identify the isolated germ cells by morphology. These features include cell intact membrane, cell volume, structure of the nucleoli and chromatin (Bellvé et al., 1977). Besides, the yield of total dissociated germ cells from different testes is another of importance factors to evaluate the dissociated cells since obviously, the cells with disrupted membrane (dead cells) exposed to enzymatic solution and mechanical condition cannot be achieved from the dissociation of the tissue. Therefore, the evaluation of the yield of germ cells, on one hand, prove the validity of the technique in cell dissociation for postmortem testis whether or not be subject to skepticism, on the other hand, prove the fragility of the germ cells.

Therefore, in this experiment, we would performed cell dissociation and counting retrieved germ cells numbers to investigate the morphological and the yield of dissociated germ cells in postmortem testis at different postmortem interval, with the purpose to evaluate the effect of deterioration from cellular level and more accurately estimate the feasibility for germ cell transplantation.

3.2 Materials and Methods

3.2.1 Enzymatic digestion of the testes isolated from postmortem fish

To compare the germ cell numbers obtained from postmortem fish, enzymatic digestion were conducted against testes isolated from each group. All the testes were isolated from 6 individuals of each incubated group. The main testicular blood vessel and mesorchium were removed with a pair of forceps under an SMZ-U stereomicroscope (Nikon, Tokyo, Japan). Then, the trimmed testes were minced and incubated in 1ml of 0.5% trypsin (Worthington Biochemical Corp., Lakewood, NJ) in PBS (pH=8.2) containing 1% fetal bovine serum (Gibco Invitrogen Co., Grand Island, NY) and 0.5% DNase I (Roche Diagnostics, Mannheim, Germany) for 2h at 20°C. During incubation, gentle pipetting was applied physically to disperse any remaining intact portions of the testis. The resultant cell suspension were filtered through a nylon screen with a pore size of 42μm (N-No.330T; Tokyo Screen Co. Ltd., Tokyo, Japan) to remove any undissociated cell clumps, and then resuspended in L-15 medium (Gibco Invitrogen Co.). To compare the germ cell numbers obtained from postmortem fish, the number of GFP-positive cells were calculated and compared in each incubated group.

3.2.2 Statistical analysis

All data were described as the means ± standard error of the mean (SEM). Statistical analyses were performed using SPSS software (Version 17.0 for Windows). Differences were analyzed by using a one-way analysis of variance and Tukey-Kramer's multiple comparisons test. P values < 0.05 were considered as significant difference.

3.3 Results

3.3.1 The morphology of germ cells retrieved from postmortem fish

To evaluate the germ cells retrieved from postmortem fish, the testes of 0, 6, 12 and 24 hpd were dissociated into single-cell suspensions and the GFP-positive-cells were observed under a fluorescent microscope (Fig. 3A). As a result, cells showing clear GFP signals were recovered in all groups. Since we previously found most of the dead cells were lysed by proteinase treatment during testicular tissue dissociation, these results suggested that cells retrieved from the postmortem fish were mostly viable (Fig. 3A).

3.3.2 The germ cell numbers retrieved from postmortem fish

To examine the number of germ cell which can be retrieved from postmortem fish, the GFP-positive-cell numbers were counted under a fluorescent microscope. The total number of GFP-positive cells divided by net testicular weight was calculated in each group. There were no significant difference in all groups, although cell number recovered from the 24-hpd group showed smaller tendency (Fig. 3B).

3.4 Discussion

In this experiment, we performed evaluation of germ cells derived from postmortem fish from two aspects, the morphology and the yield ability. Consequently, most of the singly dissociated germ cells from postmortem testes did not show obvious abnormal morphology even up to 24 hpd and the numbers of germ cells are comparable for each group. These results indicate that the enzyme digestion have no negative effect on the germ cells derived from postmortem testes, at least in the aspect of their restored yield. However, based on the result of the retrieved germ cell number and morphology, it is not enough to distinguish the retrieved germ cells are recovered or not, therefore, we need further evidence to prove their function in the future.

As we previously know that the caspase-positive germ cells reached the highest value at 12 hpd but decreased significantly at 24 hpd which implied that the death pathway may shift to necrosis that had the negative influence on the cell membrane. Theoretically, the number of germ cells would decrease since the necrotic cells or late-apoptotic cells which had some kinds of disruption of their membrane would be erased during the process of enzymatic digestion. On the contrary, there was no obviously decrease of the retrieved germ cell indicating that the cells can be reversible restored once we provided a balanced culture medium. The balanced culture medium phosphate-buffered saline (PBS) we used in the study was reported to be helpful in substantially enhanced the yield and viability of isolated spermatogenic cells (Loir and Lanneau, 1974; Romrell et al., 1976) and the morphological integrity of germ cells from 24 hpd was apparently well preserved in our experiment since they persist of the digestion of enzyme. However, according to the NCCD suggests that a cell can be defined as dead cells when any of the following criteria are met: (1) the cell has lost the plasma membrane integrity; (2) the nucleus of cells has undergone complete

fragmentation into discrete bodies (apoptotic bodies); (3) the corpse of cells have been engulfed by an adjacent cell in vivo. In particular, the morphological change like nuclear fragmentation is ATP required (Kass et al., 1996; Leist et al., 1997). ATP is also caspase activation dependent. Therefore, even though we obtained the comparable number of germ cells retrieved from different groups, the decrease of caspase-positive germ cells at 24 hpd indicated that they had high possibility remain to be regarded as dead cells. In a word, the germ cells we retrieved from postmortem fish may restored their morphology but may not be able to recover their function.

To verify the scenario, it is necessary to perform transplantation of these germ cell to evaluate their function. The germ cell once transplanted into the recipient's gonad would proliferate and different into gametes. If the germ cells have a reversible injury and remain functional after recovering from postmortem-derived injury, they may express their transplantability theoretically.

3.5 Reference

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3.6 Figure Captions

Fig 3.1 Micrograph of dissociated testicular cells derived from postmortem vasa-Gfp transgenic rainbow trout. A: Bright field (up), fluorescent (middle) and merge (down) views of testicular cells from postmortem fish. Scale bars indicate 20 μm in all panels.

Fig 3.2 The numbers of GFP-positive germs cells divided by net testicular weight (g). Values are described as means (N=3) ± SEM and bars indicate SEM. No significant difference at $P < 0.05$ was found for each group (Turkey-Kremer test).

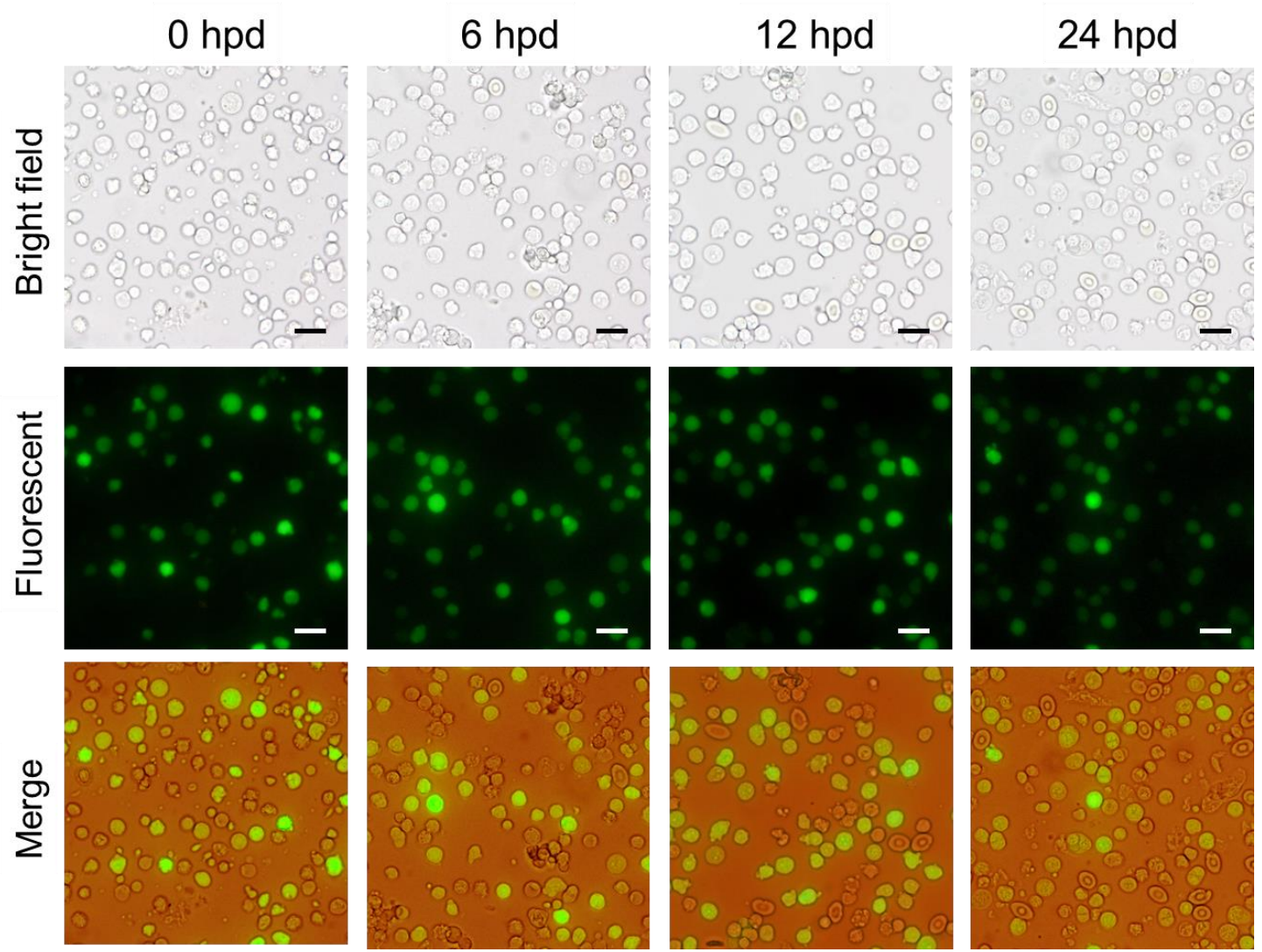

Fig 3.1 Micrograph of dissociated testicular cells derived from postmortem vasa-Gfp transgenic rainbow trout. A: Bright field (up), fluorescent (middle) and merge (down) views of testicular cells from postmortem fish. Scale bars indicate 20 μm in all panels.

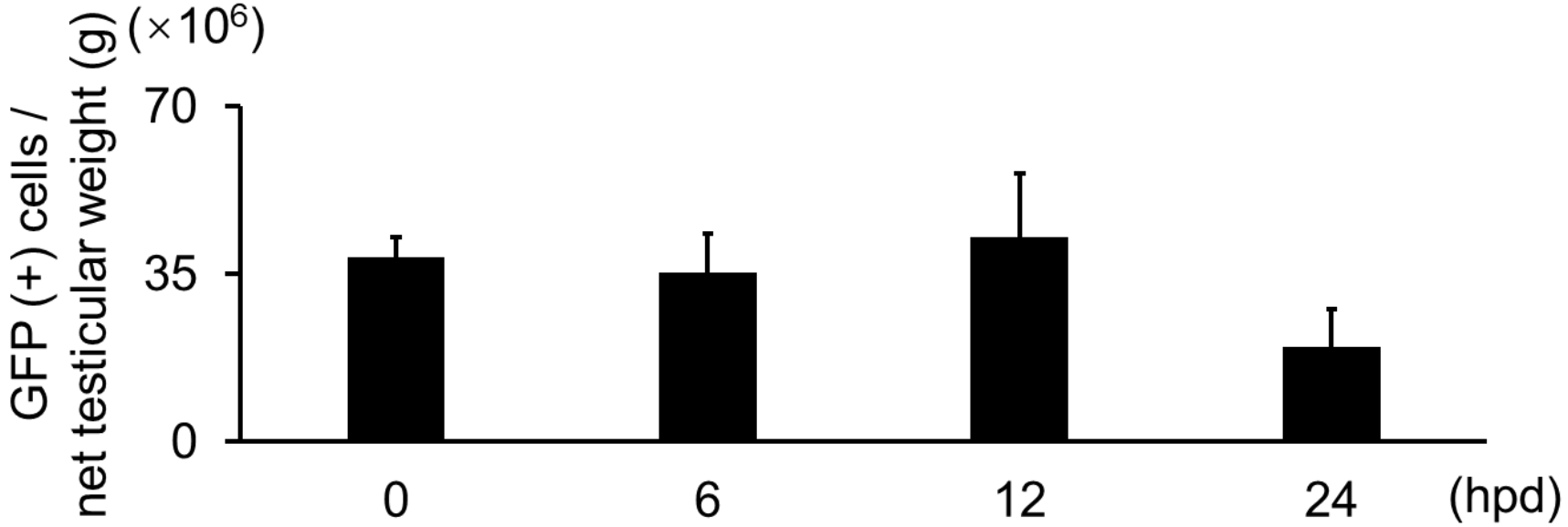

Fig 3.2 The numbers of GFP-positive germs cells divided by net testicular weight (g).

Experiment 4

Germ cell transplantation of testicular cells prepared from postmortem fish

4.1 Introduction

In order to prevent the permanent loss of the valuable genetic resources of the postmortem endangered species or breeds, it is desirable to apply germ cell transplantation system for their dissociated germ cells. However, since the germ cells derived from postmortem fish undergo deterioration, whether they can be regarded as donor is uncertain. Since our final goal is to obtain the transplantable germ cell from postmortem fish, that means the germ cells we retrieved not only maintain the function but also should have a considerable population. In the previously experiments, we evaluated the deterioration of fish and the deterioration of testes. The results indicated that no matter germ cells retrieved from postmortem fish are highly caspase-activated apoptotic cells or decreased caspase-activated necrotic cells, they all have comparable dissociated numbers and intact plasma membrane morphologically. Since the levels of ATP in the cell population would decrease as death time increase, the percentage of viable cells would also decrease. Therefore, whether all these germ cells with intact cell membrane are indeed viable and possess function should be further convinced. It is necessary to perform germ cell transplantation that provide the body environment of recipient for these deteriorated germ cells to verify their viability and function.

Germ cell transplantation has been well established to help the production of

gametes of donor species that are commercially valuable or endangered by using the recipient as a surrogate host (Okutsu et al., 2006; Yamaha et al., 2007). This technique developed due to the advantages of the germline stem cells, including primordial germ cells (PGCs) and their subsequent stages, oogonia / spermatogonia, which exhibit the migration activity and transplantability (Okutsu et al., 2006; Yoshizaki et al., 2010). The transplantability of these cells was attributed to their potency that can undergo proliferate and differentiate into functional gametes and hence contribute to transmit their genetic resources to the next generation (Brinster, 2002; Okutsu et al., 2006b). Therefore, once the germ cells with transplantability were isolated and transplanted into the peritoneal cavity of newly hatched larvae, they will undergo continued gametogenesis until produced functional sperm or eggs in male and female recipient, respectively. However, in this research, the major obstacles in the feasibility of germ cell transplantation are the numbers of viable germ cells and whether the function of transplantabilty of germ cells get lost during deterioration in vivo or during dissociation from postmortem fish. On the other hand, if the germ cells we dissociated and transplanted into the recipient can be manifested with function that they undergo the series process of colonization, proliferation and differentiation behaviors without any problem, it in turn verifies that these germ cells are viable and still possess itself the function of stem cells and transplantability.

Meanwhile, the large number of cells directly microinjectd into the body cavity of recipient can counteract the potential low migration ability of spermatogonia in response to chemotactic signals (Yoshizaki et al., 2010). That means, microinjecting the large number of viable ASG derived from postmortem fish on the other hand can guarantee the effective of transplantation. Since we dissociated considerable numbers of germ cells from different groups according to the result of Exp. 3, therefore, by injecting the same large amounts of cells derived from different postmortem fish can effectively compare the viability and

transplantability of these germ cells that have considerable dissociated numbers.

Therefore, in this experiment, we performed germ cell transplantation for the germ cells derived from postmortem fish through injecting them into the body cavity of the recipient and compare the viability and transplantability by evaluating the transplantation efficiency of injected recipient. Thought this experiment, we could evaluate the function of germ cells derived from postmortem fish and finally correlate the deterioration of the postmortem fish, testes with the feasibility for germ cell transplantation.

4.2 Materials and Methods

4.2.1 Germ cell transplantation of testicular cells prepared from postmortem fish

To investigate the transplantability of germ cells retrieved from postmortem fish, germ-cell transplantation were conducted. Approximately10,000 of GFP-positive germ cells which were collected from 0, 6, 12 and 24 hour-post-death (hpd), were transplanted into the peritoneal cavity of newly hatched larvae of wild-type rainbow trout at 30 days-post-fertilization (dpf). The transplantation procedure was followed by accordance with previously described by Okutsu et al (2006). Transplanted recipients were dissected at 25, 90, 150 dpt and then observed to confirm whether the recipient genital ridges possess GFP-positive cells or not. The transplantation efficiency of donor-derived germ cells in the recipient genital ridges was calculated by the following formula: Transplantation efficiency (%) = number of fish incorporating fluorescent cells in genital ridges / number of fish observed × 100.

4.2.2 Statistical analysis

All data were described as the means ± standard error of the mean (SEM). Statistical analyses were performed using SPSS software (Version 17.0 for Windows). Differences were analyzed by using a one-way analysis of variance and Tukey-Kramer's multiple comparisons test. P values < 0.05 were considered as significant difference.

4.3Results

4.3.1 Transplantation of spermatogonia retrieved from postmortem fish

To investigate whether ASG retrieved from dead fish maintain the ability to be incorporated into recipient gonads, 10^4 cells were intraperitoneally transplanted into larvae of wild-type rainbow trout. At 25 dpt, recipients were dissected, and their genital ridges were observed under a fluorescent microscope (Fig. 4.1). As a result, we clearly observed that the transplanted ASG had been incorporated into genital ridges of the recipients (Fig. 4.1). The transplantation efficiency of recipients which carried donor derived ASG in their genital ridges were quantified (Fig. 4.2). The transplantation efficiency of 0, 6, 12, and 24 hpd were 86.29 ± 5.70%, 82.22 ± 11.76%, 73.33 ± 3.33%, and 6.68 ± 6.66%, respectively. The transplantation efficiency of 24 hpd was significantly lower than that of the other groups. The incorporated GFP-positive-cell numbers of genital ridges from 0, 6, 12, and 24 hpd were 10.88 ± 4.00, 11.02 ± 3.80, 6.45 ± 1.91 and 1.17 ± 0.60, respectively (Fig. 4.3). The tendency that the incorporated GFP-positive-cell numbers were decreasing in a time-dependent manner could be seen. There were significant difference between 24 hpd group and the others.

4.3.2 Proliferation and differentiation of donor spermatogonia retrieved from postmortem fish in the recipient gonads

To further confirm whether the incorporated cells from postmortem fish could proliferate and differentiate in the recipient gonads, the whole gonads of recipients at 90 and 150 dpt were isolated and observed under a fluorescent microscope (Fig. 4.4 and 4.6). Large

cell clusters showing green fluorescence were observed in both testes and ovaries isolated from 0-12 hpd fish (Fig. 4.5 and 4.7). Since dead cells often shows autofluorescense, immunohistochemistry was performed using anti-GFP antibody to make sure the green fluorescence detected in the recipient gonads were surely caused by GFP. As a result, these gonadal sections were clearly positive for anti-GFP antibody (Fig. 4.5 and 4.7), indicating that the postmortem fish-derived ASG surely proliferated in recipient gonads. In addition, these analyses revealed that postmortem fish-derived ASG were differentiated into oocytes possessing diameter of more than 100 μm and further growing in the female recipients (Fig. 4.5 and 4.7). In the recipients receiving the ASG retrieved from fish of 24 hpd group, only one specimen possessing GFP-positive oocytes in their gonads were observed at 90 dpt. These results suggested that ASG retrieved from fish of 24 hpd group barely contained viable ASG, which can differentiate normally into female germ cells in the genital ridges of the recipients. In 90 dpt, the frequency of recipients which carried GFP-positive colonies of germ cells derived from fish of 0, 6, 12, and 24 hpd groups were 77.77 ± 14.70%, 77.77 ± 5.53%, 55.57 ± 5.57%, and 5.56 ± 5.56%, respectively (Fig. 4.8). In 150 dpt, the frequency of recipients which carried GFP-positive colonies of germ cells derived from fish of 0, 6, 12, and 24 hpd groups were 75.70 ± 9.74%, 78.23 ± 13.71%, 53.9 ± 8.40%, and 0%, respectively (Fig. 4.9). No significant difference of these values were found among 0, 6, 12 hpd at both 90 and 150 dpt.

4.4 Discussion

In the present study, we demonstrated whether the germ cells derived from postmortem fish are applicable to be used as donor cells for germ cell transplantation. As a result, transplanted ASG from no more than 12 hpd postmortem fish differentiated successfully and underwent either oogenesis or spermatogenesis in the ovary and testis of recipient, respectively. It was worth noting that the transplantability of ASG retrieved from postmortem trout did not decreased for as long as 12 hours pd in the running water which was 10.5℃. On the other hands, although, ASG derived from 24 hpd fish still possessed the ability to be incorporated and differentiated in the genital ridges of the recipients, their transplantation efficiency was low. Only one specimen possessing GFP-positive oocytes in their gonads were observed at 90 dpt. It suggested that ASG retrieved from postmortem fish up to 24 hpd were confirmed to proliferate and start differentiation into oocytes without any problem. These results suggested that ASG retrieved from postmortem fish are still alive and maintain the transplantability and differentiation ability.

In germ cell transplantation of teleost, as we mentioned before that only undifferentiated germ cells including primordial germ cells, ASG and oogonia can be incorporated into recipient gonads after transplantation into recipient body cavities and can differentiate into functional eggs and sperm, depends on the recipient sex (Yoshizaki and Lee, 2018; Yoshizaki and Yazawa, 2019). Importantly, transplanting the spermatogonia into female and male recipients makes it possible to obtain donor-derived functional eggs or sperm, respectively. It means that these undifferentiated germ cells possess stem cell activities and sexual plasticity (Okutsu et al., 2006). Although, above-mentioned three types of germ cells in each can be used as donor, our laboratory has preferentially focused on ASG transplantation because it is easier to obtain enough numbers of transplantable germ cells,

compared with primordial germ cells and oogonia for practical uses. In primordial germ cells, they can only be harvested from newly hatched larvae (Takeuchi et al., 2002). Further, the proportion of oogonia contained in dissociated immature ovary (5.20 ± 0.90%) is notably lower compared with those of ASG dissociated from same aged males (49.76 ± 7.25%) in rainbow trout (Ichida et al., 2019). In this study, the postmortem male fish were proved to be used as donor; however, the accidental death occur randomly to both females and males in captive breeding. Moreover, in case of protogyny and protandry species, it is possible that the rearing population are all females. Therefore, if they accidentally died, it is impossible to obtain testes used for donor ASG preparations. Therefore, confirming the feasibility of oogonia isolated from postmortem female fish are important future task for spreading this strategy to commercial farm and research institutions, having valuable fish species or strains.

In the present study, it was demonstrated that ASG retrieved from 24 hpd postmortem rainbow trout left at 10.5℃ possess low transplantability. This result indicated that the biological conditions of germ cells retrieved from over 12 hpd postmortem fish had been decreased. However, there is no significant difference in transplantation efficiency between 0-12 hpd. Confirming the reproduction of functional eggs and sperm derived from postmortem trout up to 12 hpd from recipient to produce offspring will be future task. Meanwhile, in aquaculture, for successful germ cell transplantation using ASG retrieved from postmortem fish, periodical checking of their target fish with no more than 12 hours interval is preferable in order to obtain testes still carrying enough numbers of transplantable ASG.

In conclusions, ASG retrieved from rainbow trout within 12 hours after their death, are proven to maintain high transplantability and to undergo successful differentiation in recipient gonads after their transplantation. Thus, germ cell transplantation system using postmortem fish developed in this study enables it to pave the way toward production of

offspring derived from the dead fish.

4.5 Reference

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4.6 Figure Captions

Fig 4.1 Micrograph of recipient genital ridges at 25 days after transplantation (dpt) with germ cells derived from postmortem *vasa-Gfp* transgenic rainbow trout. Bright field (left) and merge with fluorescent (right) views of recipient genital ridges. The dotted red lines indicate the location of genital ridge. Scale bars indicate 20μm in all panels.

Fig 4.2 Transplantation efficiency indicates the frequency (%) of recipients possessing GFP-positive cells in their genital ridges out of total recipients checked.

Fig 4.3 Incorporated GFP-positive germ cell numbers in genital ridges of recipients. Values are described as means (N=3) ± SEM and bars indicate SEM. Different letters indicate significant difference at $P < 0.05$ (Turkey-Kremer test).

Fig 4.4 Micrograph of the recipient gonads isolated from 90 dpt recipients. Bright and merged with fluorescent views of whole gonad of recipients. Scale bars indicate 100 μm.

Fig 4.5 Immunohistochemistry result of recipient gonads isolated from 90 dpt recipients. Fluorescent and merged with bright views of each gonadal section after immunohistochemistry (IHC) with anti-GFP antibody. HE staining against the same sections of IHC. Scale bars indicate 20 μm.

Fig 4.6 Micrograph of the recipient gonads isolated from 150 dpt recipients. Bright and merged with fluorescent views of whole gonad of recipients. Scale bars indicate 100 μm.

Fig 4.7 Immunohistochemistry result of recipient gonads isolated from 150 dpt recipients. Fluorescent and merged with bright views of each gonadal section after immunohistochemistry (IHC) with anti-GFP antibody. HE staining against the same sections of IHC. Scale bars indicate 20 μm.

Fig 4.8 Transplantation efficiency at 90 dpt. Values are described as means (N=3) ± SEM and bars indicate SEM. Different letters indicate significant difference at $P < 0.05$ (Turkey-Kremer test).

Fig 4.9 Transplantation efficiency at 150 dpt. Values are described as means (N=3) ± SEM and bars indicate SEM. Different letters indicate significant difference at $P < 0.05$ (Turkey-Kremer test).

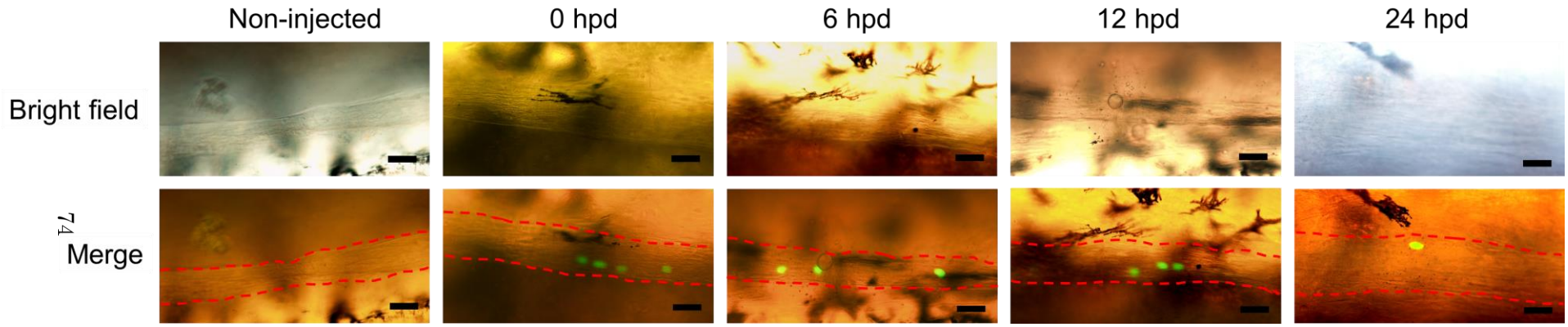

Fig 4.1 Micrograph of recipient genital ridges at 25 days after transplantation (dpt) with germ cells derived from postmortem *vasa-Gfp* transgenic rainbow trout.

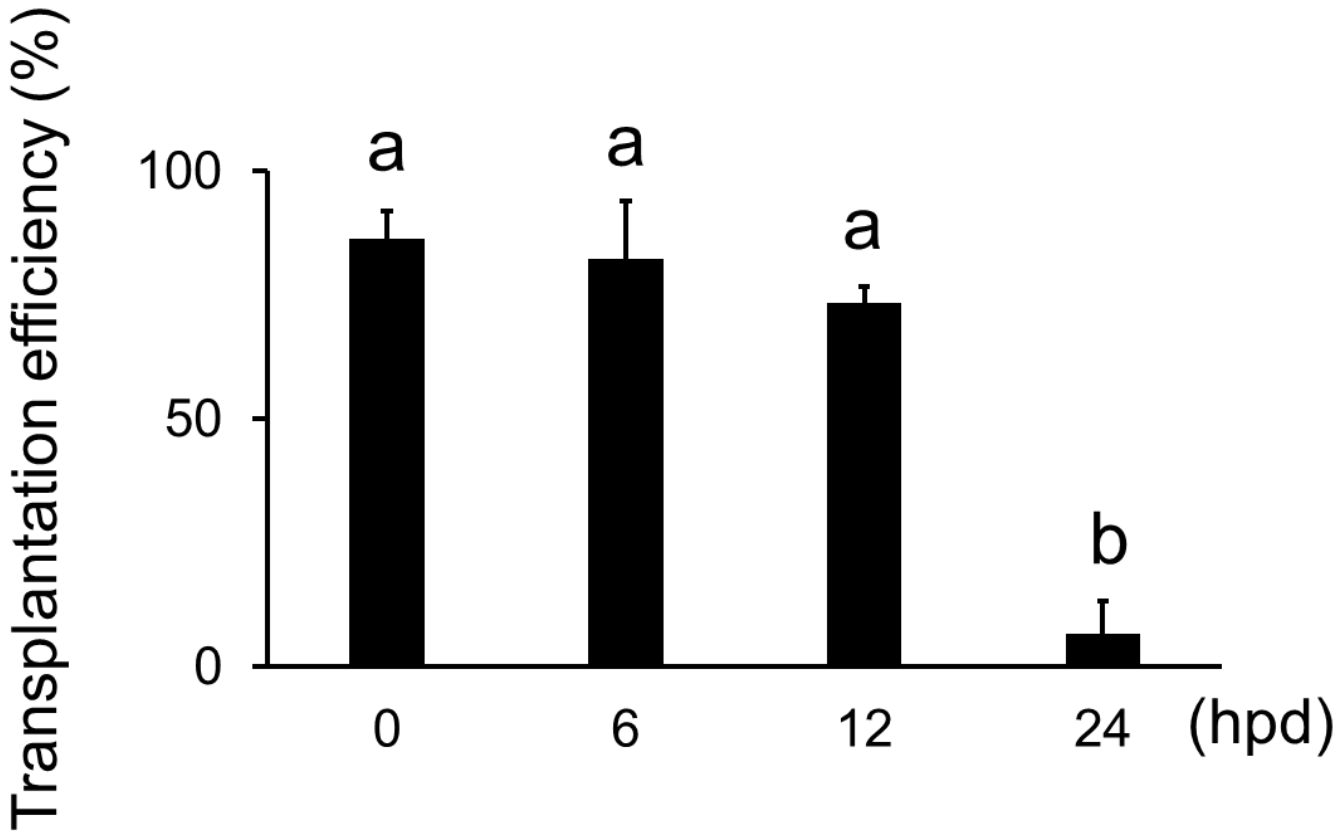

Fig 4.2 Transplantation efficiency indicates the frequency (%) of recipients possessing GFP-positive cells in their genital ridges out of total recipients checked.

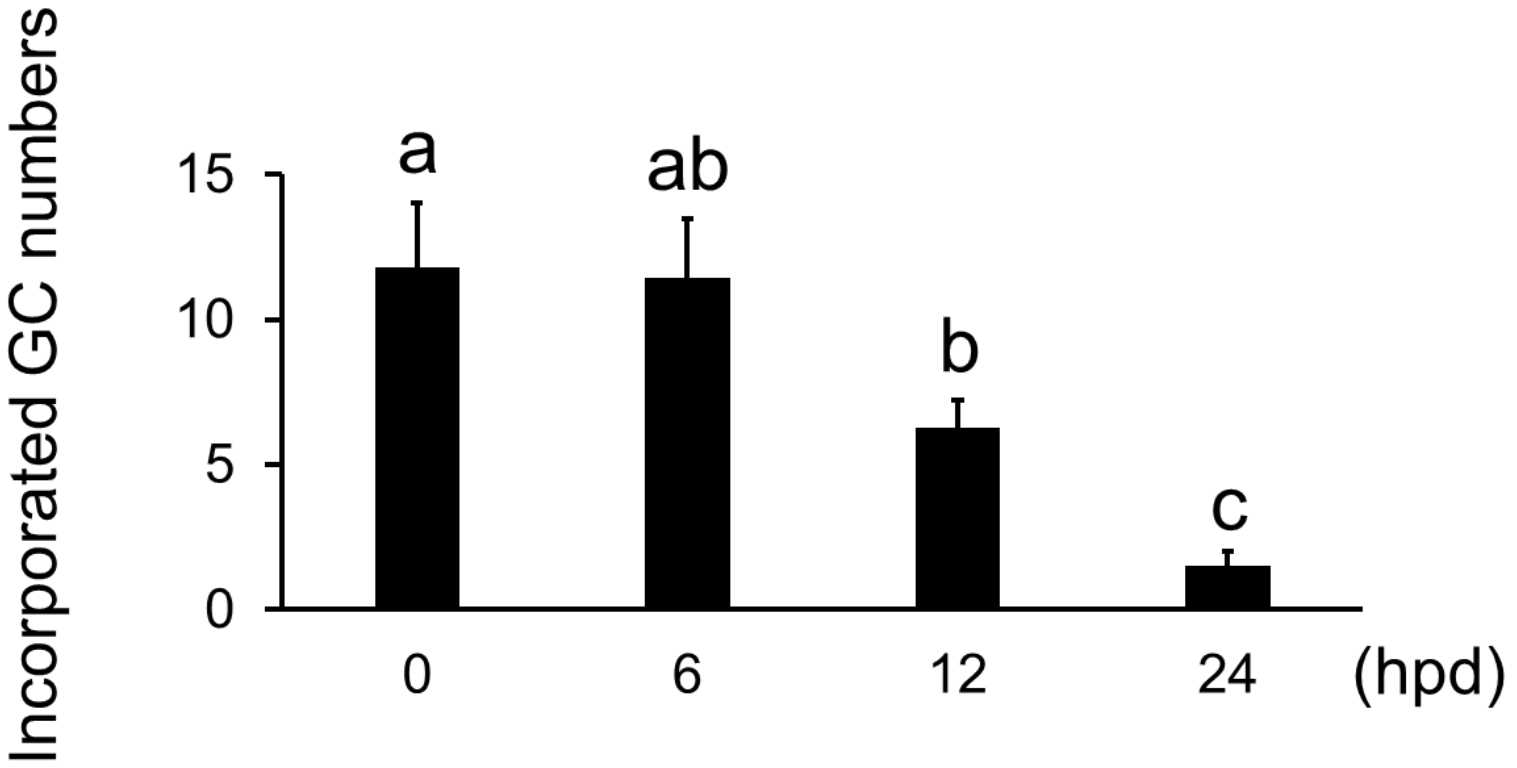

Fig 4.3 Incorporated GFP-positive germ cell numbers in genital ridges of recipients.

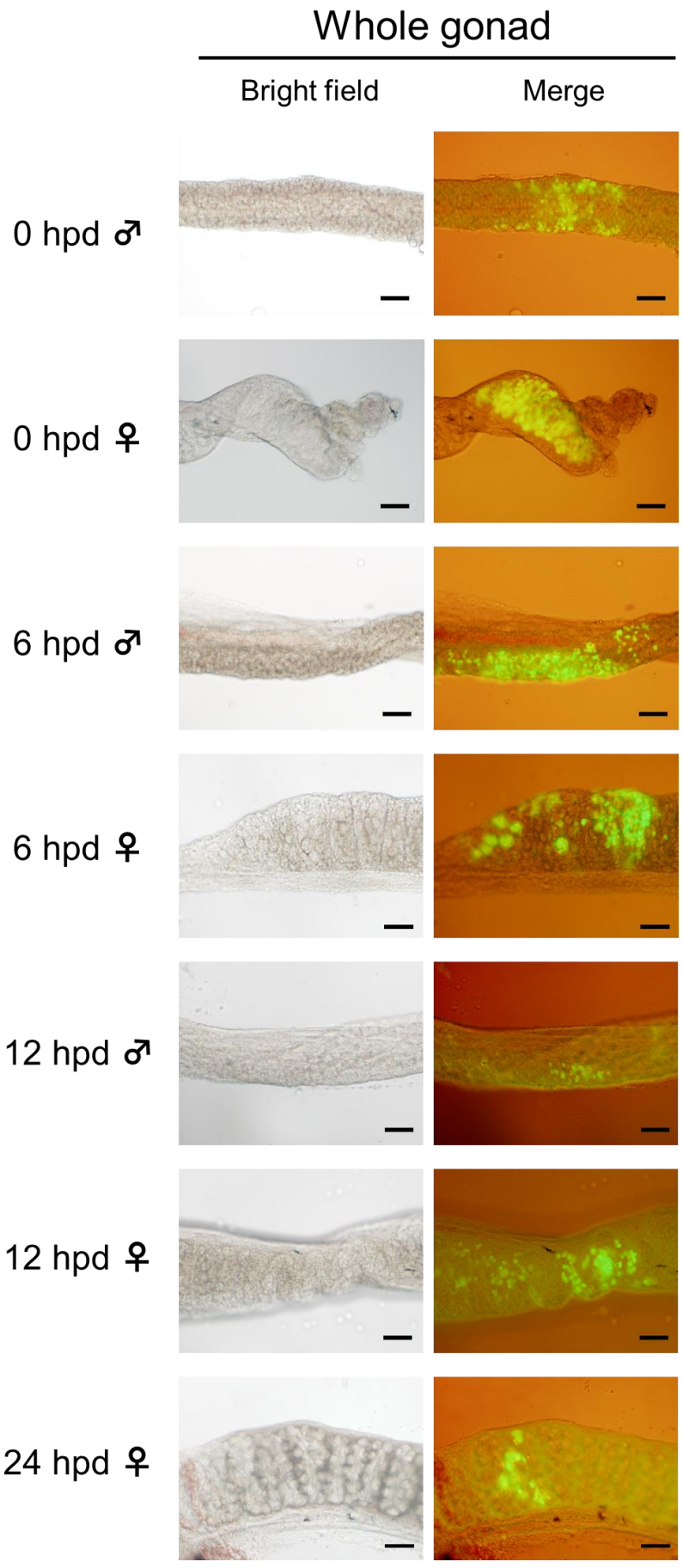

Fig 4.4 Micrograph of the recipient gonads isolated from 90 dpt recipients. .

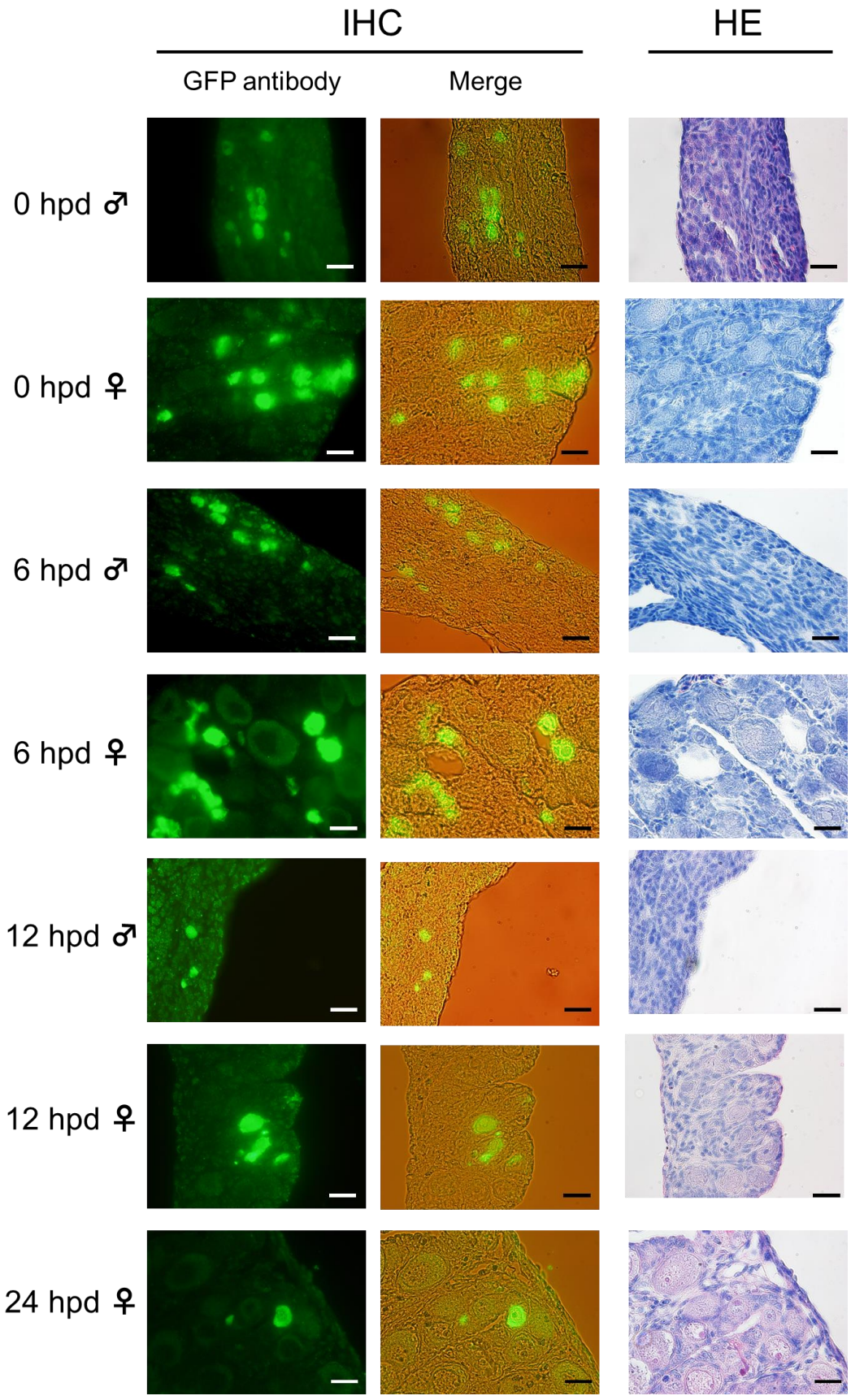

Fig 4.5 Immunohistochemistry result of recipient gonads isolated from 90 dpt recipients.

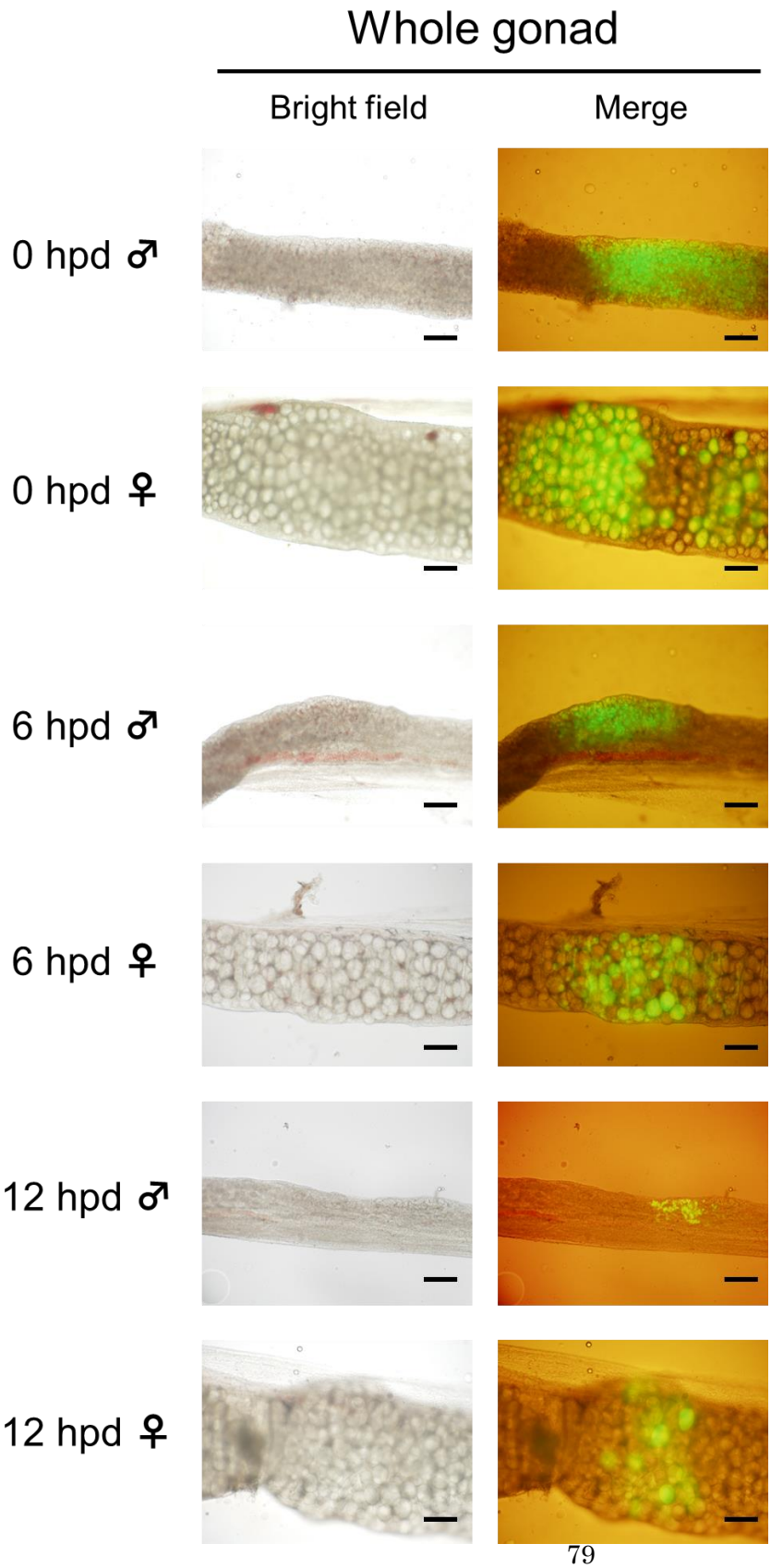

Fig 4.6 Micrograph of the recipient gonads isolated from 150 dpt recipients.

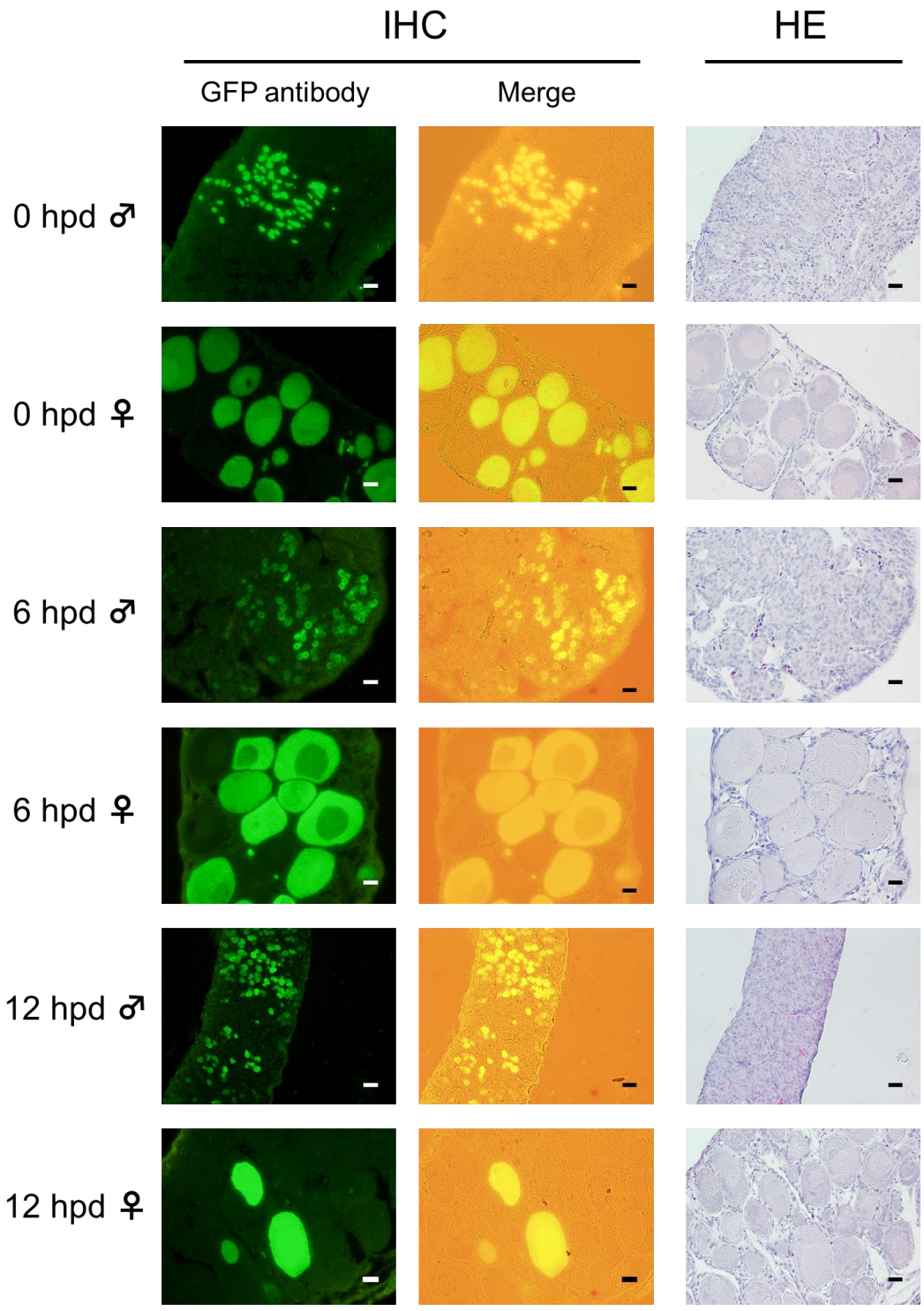

Fig 4.7 Immunohistochemistry result of recipient gonads isolated from 150 dpt recipients.

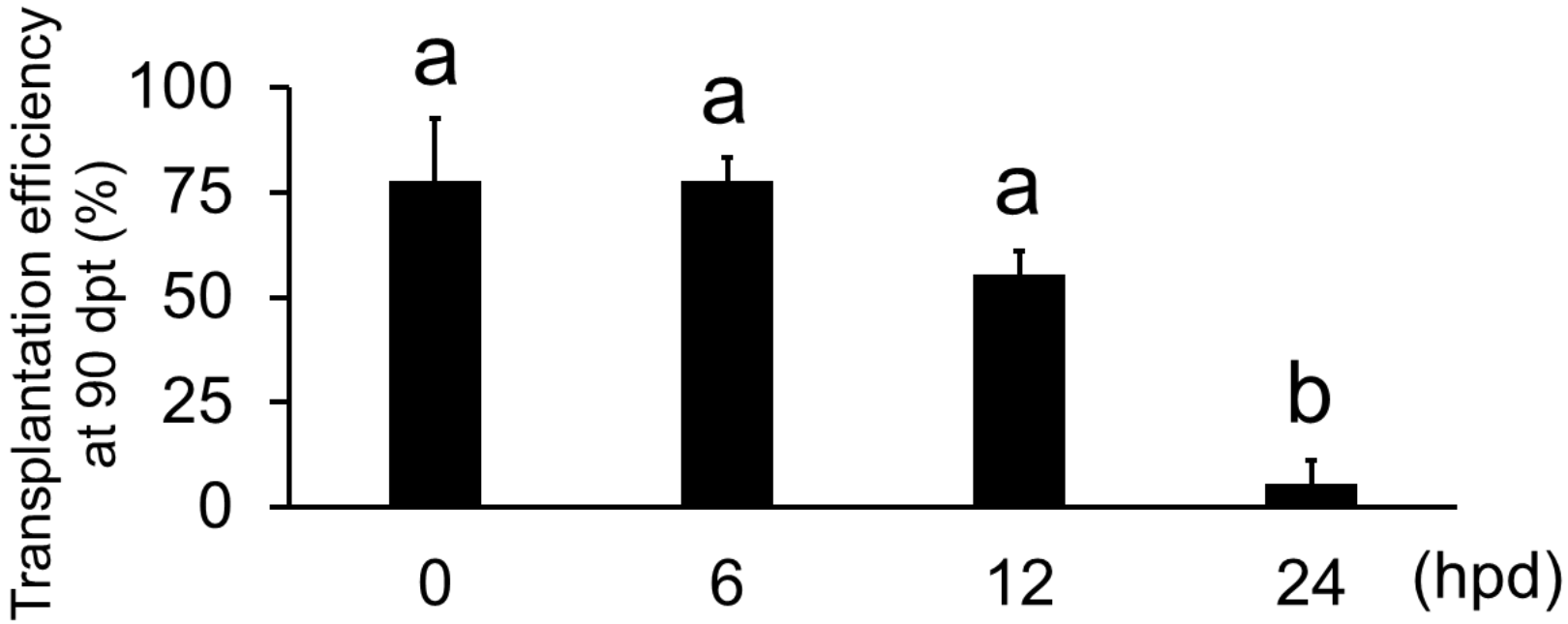

Fig 4.8 Transplantation efficiency at 90 dpt.

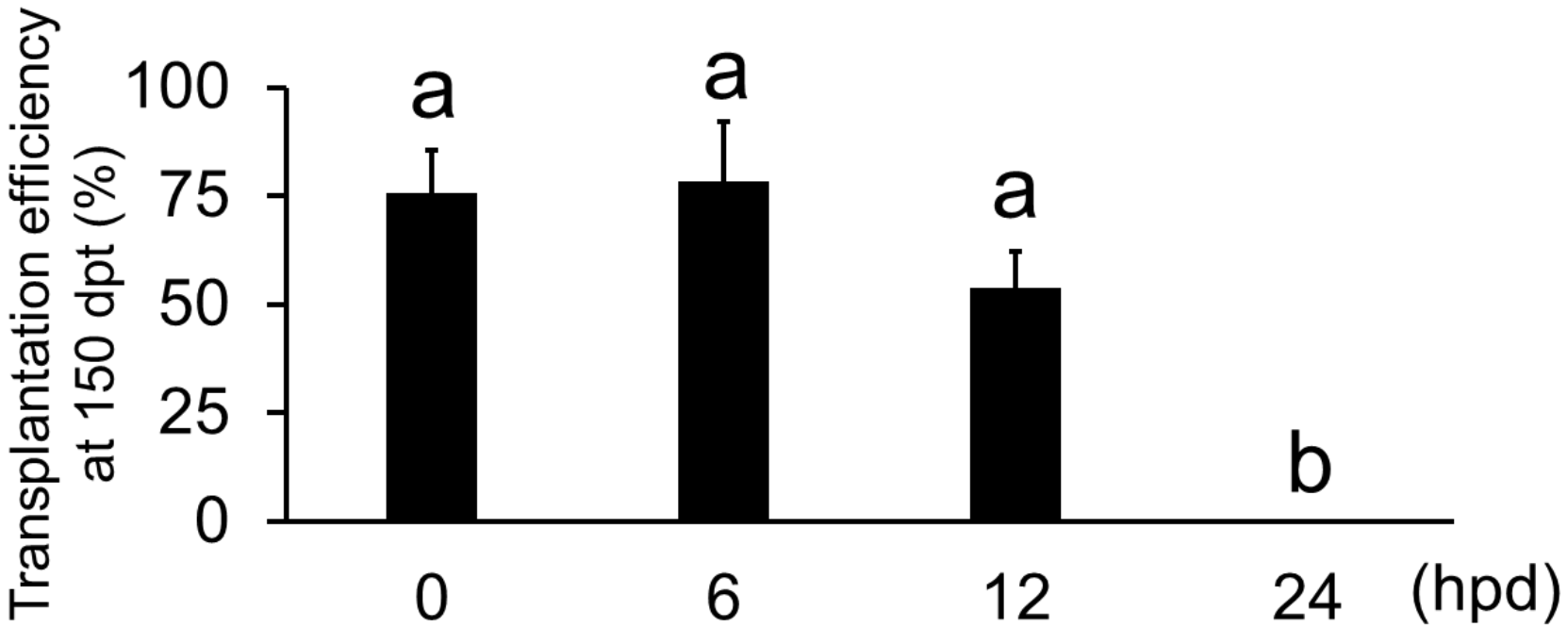

Fig 4.9 Transplantation efficiency at 150 dpt.

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