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死後のニジマスをドナーに用いた生殖細胞移植系の開発

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［課程博士・論文博士共通］

博士学位論文内容要旨
Abstract

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論文題目 Title	Development of germ cell transplantation system using postmortem rainbow trout as a donor		

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, 10^4 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 was 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 was 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.