Deliverable D5.2
Report on preparation of specific recipients for GSC transfer

WP 5
Deliverable D5.2
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1. Objective

The main objective of this deliverable is to prepare appropriate recipients for transplantation of germ cells and surrogate production. We compare recipients treated with different sterilization methods and compare their reproductive performance.

2. Background

Germ stem cell (GSC) manipulation offers a cutting-edge technique in fish reproductive biotechnology. GSCs from one fish can be transplanted into another, potentially of a different species. These cells can migrate, colonize the genital ridge, and even undergo trans-differentiation, where they adapt based on their new environment, ultimately leading to donor-derived gametes (Goto and Saito, 2019). This process is supported by cryopreservation procedures that allow efficient preservation and recovery of both male and female GSCs (Franek et al., 2019a,b). Recent advancements have even enabled the production of genetically edited gametes, bypassing the potential non-viability issues in adult fish due to mutations (Zhang et al., 2020, 2021).

Surrogate reproduction is gaining traction for species conservation and aquaculture, especially with the increasing research on GSC manipulation. GSC transplantation can speed up gametogenesis in larger, late-maturing species by using smaller, early-maturing recipients. Additionally, surrogacy can enhance gamete production by transplanting GSCs from smaller species to larger, more fecund ones (Nayak et al., 2023). The potential of this technology also extends to improving breeding strategies by dispersing superior germplasm through surrogates.

However, maximizing the success of this technology requires optimization. Factors like the optimal time for GSC harvesting, conditions for isolation, purification, and in vitro expansion have been identified. Research has shown that the age of the recipient and the number of transplanted spermatogonia can affect the colonization rate. There is also a limited time for GSCs to integrate into the host's genital ridge, pointing to certain biological constraints in the process. Factors like the genetic relationship between the donor and recipient, the use of sterile versus unsterile recipients, and sterilization methods have all been studied to understand their impact on the colonization rate and gamete production (Franěk et al 2022).

Sterilization is essential for effective surrogacy, ensuring that introduced GSCs do not face competition in the gonads. Fish can be sterilized in various ways, from germ cell-free methods to fully developed yet non-functional gonads. Techniques include targeting the dead end (dnd) gene responsible for germ cell migration and maintenance (Baloch et al., 2019a). While effective, gene editing for sterilization is complex and requires precision. There are also regulatory considerations given the genetic modifications involved. Another approach is triploidization, which alters chromosome number, leading to impaired gametogenesis (Piferrer et al., 2009). While this method is suitable for mass production, it's not universally effective. Hybridization, another sterilization method, involves merging two species, often leading to compromised reproductive capabilities due to genetic mismatches (Fujimoto et al., 2008; Tichopad et al., 2020).

In summary, GSC manipulation in fishes is a promising frontier in reproductive biotechnology. As research progresses, refining techniques and understanding variables will be crucial to harnessing its full potential for species preservation and advanced aquaculture practices.

3. Methodology

The study was conducted at the Faculty of Fisheries and Protection of Waters (FFPW), University of South Bohemia in Ceske Budejovice, Vodnany, Czech Republic. The facility has the competence to perform experiments on animals (Act no. 246/1992 Coll., ref. number 16OZ19179/2016–17214). The Institutional Animal Care and Use Committee of the FFPW approved the methodological protocol of the current study according to the law on the protection of animals against cruelty (reference number: MSMT-6406/2019–2).

Fish and Production of Recipients

Zebrafish broodstock maintenance followed established protocols. The study involved three zebrafish lines: AB line, vas:EGFP line, and pearl danio. All experimental groups underwent *in vitro* fertilization. The experiments aimed to create different recipient groups using eggs and sperm from the same parents. One group of fish, called the MO group, was depleted of primordial germ cells by injecting them with *dnd1* antisense morpholino oligonucleotide. Another group was made triploid through a heat-shock process. Hybrid zebrafish (H) were also created using females from the zebrafish line and males from the pearl danio line. These embryos were cultivated at a consistent temperature of 28°C. After five days post fertilization, they were fed with Paramecium sp. and later with Artemia sp. Four weeks post-fertilization, they were moved to a specialized zebrafish housing system.

Preparation of Donor Cells and Transplantation

Adult zebrafish from the vas::EGFP line were used as donors, where germ cells express EGFP. After being anaesthetized, their testes were removed and treated to extract spermatogonia. This involved cutting the testes, washing them, and then digesting them with specific media. The resulting suspension was then prepared for transplantation. Transplantation occurred five days post fertilization. Recipient fish were anaesthetized, and cells from the donor were injected into them. The aim was to ensure that every recipient received an approximate and consistent number of cells. Four different transplanted groups were established (intact, MO, 3n, and H), and controls were set up for each group.

Identification of Germline Chimeras

Two weeks after the transplantation, the fish were checked for EGFP signals to identify germline chimeras. Adult fish that survived were prepared for sperm collection, and the sperm was examined for the EGFP signal. DNA extraction and PCR techniques were used for further confirmation.

Reproductive Performance of Germline Chimeras

Both male germline chimeras and their control counterparts were placed in spawning tanks with females from a control group. Several groups were further studied for progeny survival, checking various stages like fertilization rate, hatching rate, and swim-up rate. DNA tests were conducted on some larvae for further analysis.

For in-vitro fertilization, sperm from selected males was pooled and used to fertilize eggs from control females. Additionally, attempts were made to spawn EGFP positive females from a specific group with control males. The outcome, in terms of egg fertilization and hatching rates, was closely monitored.

4. Results and Discussion

Survival, transplantation success, and colonization patterns

The lowest survival during recipient production was noted in the MO and 3n groups due to injection into embryos and heat shock treatment, respectively. After transplantation, survival in the MO transplanted group matched controls, while the 3n, H transplanted, and control groups were slightly lower. Overall survival ranged from 65 to 85% in all groups up to 6 months of age. Two weeks post-transplantation, consistent outcomes were observed across various sterilization methods. EGFP positive cells were primarily found in the posterior or medial sections of the body cavity, with a few in the anterior.

Gonadal development in sterilized controls and surrogates

Sterilization treatments led to varied gonadal development patterns. MO treated fish had small testes without germ cells, while triploids had gonads with all spermatogenic stages but with defects in meiosis. In contrast, hybrid males displayed three distinct gonadal phenotypes. First hybrid gonadal phenotype had well-developed testes with few abnormally sized spermatozoa which was later observed with a light and electron microscope. Second phenotype had undeveloped testes lacking germ cells and few individuals showed combinations of one developed and one undeveloped testis. Hybrid females had ovaries mainly composed of larger cells, indicating early stage meiotic oocytes. Only a few hybrid females were identified among all adult surviving hybrids. The incidence of adult germline chimeras was highest in the MO transplanted group, followed by the 3n transplanted group. Testicular development varied across the sterilization treatments.

Transplanted GSCs' performance

Transplanted GSCs managed to reconstitute spermatogenesis in all tested sterilization methods. A complete sterilization (MO) promoted exogenous germ cell development. Triploid and non-sterilized recipients showed competition between introduced GSCs and endogenous germ cells. AB germline chimeras displayed an uneven distribution of exogenous germ cells. Histological analysis found testes with similar morphology to controls in 3n transplanted and H transplanted groups. Some females from the AB T (transplanted non sterilized fish) group showed EGFP signals in their ovaries, confirming the presence of exogenous oogenesis from donor-derived male GSCs.

Reproductive performance of chimeric males

The sperm concentration and total amount in germline chimeras were influenced by the smaller size of testes comprising donor-derived germ cells. MO recipients had the highest sperm amount and concentration among sterilized recipients. All sterilization methods affected sperm motility and velocity, with results varying among groups.

The study focused on assessing various fish sterilization methods using a zebrafish model and the resulting reproductive impact. The choice of recipient fish is influenced by availability and survival, which correlates with the effort needed for sterilization. Methods like hybridization and triploidization are preferred for inducing sterility on a large scale. Each sterilization technique has advantages and drawbacks. Sterilization can affect survival rates prior to transplantation, with heat shock and MO injection notably reducing survival. However, post-transplantation survival is more critical, as it determines the success of the introduced cells. All tested recipients, including non-sterilized ones, had gonads that supported the transplanted cells. However, early colonization rates post-transplantation did not always predict successful long-term results. The study revealed that treatments like PGCs depletion and triploidization enhanced the development of chimeric gonads in adult fish. Notably, females from the AB T group produced donor-derived eggs, a first in zebrafish research. However, the number of donor-derived eggs was limited. Overall, the study underscores the need for careful consideration when selecting sterilization methods, taking into account both immediate and long-term reproductive outcomes.

5. Conclusion

GSC manipulation is a powerful biotechnology to ameliorate breeding of aquaculture species and preserve valuable genetic resources in environmentally relevant or even endangered species. This study aimed to compare the strengths and weaknesses of different sterilization treatments in zebrafish, an essential factor influencing the adult germline chimera induction rate and their reproductive performance. Of the utmost importance was that germ cell-free gonads with normal somatic cells were identified as the best environment for transplanted cells, yielding the highest adult germline chimera rate and gonadal development. Importantly, reproductive performance of males including sperm quantity and motility parameters and fertilization rate clearly favours germ cell depleted recipients. The use of triploid and hybrid males from the point of view of producing sufficient quantity and quality sperm proved to be risky to achieve stable results. Moreover, only germ cell depleted recipients retained reproductive characteristics of the donor strain. The present findings should help in deciding what type of sterilization should be used prior to transplantation and surrogacy induction, especially in non-model fish species. The overall suitability and versatility of the zebrafish surrogate model can be utilized to provide further insights into the mechanism of GCs behaviour in the recipient's gonads and isolate specific factors influencing promotion of the exogenous GCs development. Our interest is also directed to the molecular aspects of surrogacy. Nowadays, GSCs manipulations and surrogacy are performed on wide range of species. However, little is known about the lifetime or transgenerational consequences of gametes produced from surrogate parents and how they can possibly influence resulting progeny and their performance.

6. Appendix

A significant part of the deliverable was published here: Franěk, R., Cheng, Y., Fučíková, M., Kašpar, V., Xie, X., Shah, M.A., Linhart, O., Šauman, I. Pšenička, M. 2022. Who is the best surrogate for germ stem cell transplantation in fish? Aquaculture, Volume 549, 737759, ISSN 0044-8486, https://doi.org/10.1016/j.aquaculture.2021.737759. (IF 2022: 5.135, AIS 2022: 0.635).

7. References

Baloch, A.R., Franek, R., Saito, T., Martin, Psenicka, 2019a. Dead-end (dnd) protein in fish — a review. Fish Physiol. Biochem. 47, 777–784. https://doi.org/10.1007/s10695-018-0606-x.

Franěk R, Marinović Z, Lujić J, et al (2019a) Cryopreservation and transplantation of common carp spermatogonia. PLOS ONE 14:e0205481. doi: 10.1371/journal.pone.0205481

Franěk R, Tichopád T, Fučíková M, et al (2019b) Production and use of triploid zebrafish for surrogate reproduction. Theriogenology 140:. doi: 10.1016/j.theriogenology.2019.08.016

Fujimoto, T., Yasui, G.S., Yoshikawa, H., Yamaha, E., Arai, K., 2008. Genetic and reproductive potential of spermatozoa of diploid and triploid males obtained from interspecific hybridization of

Misgurnus anguillicaudatus female with M. mizolepis male. J. Appl. Ichthyol. 24, 430–437. https://doi.org/10.1111/j.1439-0426.2008.01131.x.

Nayak, R., Franěk, R., Šindelka, R., Pšenička, M., 2023. Enhancement of zebrafish sperm production via a large body-sized surrogate with germ cell transplantation. Communications Biology 6: 412.

Piferrer F, Beaumont A, Falguière JC, et al (2009) Polyploid fish and shellfish: Production, biology and applications to aquaculture for performance improvement and genetic containment. Aquaculture 293:125–156. doi: 10.1016/j.aquaculture.2009.04.036

Tichopad, T., Vetesnik, L., Simkova, A., Rodina, M., Franek, R., Psenicka, M., 2020. Spermatozoa morphology and reproductive potential in F1 hybrids of common carp (Cyprinus carpio) and gibel carp (Carassius gibelio). Aquaculture 521, 735092.

Zhang, F., Li, X., He, M., Ye, D., Xiong, F., Amin, G., Sun, Y., 2020. Efficient generation of zebrafish maternal-zygotic mutants through transplantation of ectopically induced and Cas9/gRNA targeted primordial germ cells. J. Genet. Genomics 47, 37–47. https://doi.org/10.1016/j.jgg.2019.12.004.

Zhang, F., Li, X., Hao, Y., Li, Y., Ye, D., He, M., Sun, Y., 2021. Surrogate production of genome edited sperm from a different subfamily by spermatogonial stem cell transplantation. bioRxiv**.** **https://doi.org/10.1101/2021.04.20.440715, 2021.04.20.440715.**

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