In vitro culturing of male Danio rerio germ cells

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Note: This report details my work performed at the National Institute of Genetics in Mishima Japan in Dr. Noriyoshi Sakai's lab. My time spent in Dr. Sakai's lab was possible thanks to the National Science Foundation, NSF, funded program: "IRES: Summer Biology Research Program in Japan". The report is entirely my original work, with no external contributions. I received ongoing support, guidance, and advice from the other authors throughout my time at NIG. The report is based on their prior research findings.

Abstract

Aquaculture plays a crucial role in Japan and globally, as fish are a vital source of nutrition for many people. However, some fish species require a prolonged period of time before they reach sexual maturity, and thus aquaculture in some species remains challenging. By identifying the conditions necessary to culture germ cells, it may be possible to isolate them from the endocrine system and its complex interplay of hormones and accelerate their development. This report details the successful development of a spermatogonial culture system capable of producing functional sperm, which was subsequently used to create embryos through artificial fertilization.

Introduction

Aquaculture is a crucial tool used globally to sustain various fish populations for both conservation and consumption. Despite ongoing innovations aimed at creating cleaner fisheries, many species remain difficult or nearly impossible to culture due to their prolonged developmental periods. For instance, the Bluefin Tuna, a widely sought-after species, takes five years to reach sexual maturity (Corriero et al., 2019). Selective breeding, an important practice in aquaculture, is used to produce stronger, larger, and potentially more desirable fish. However, the effectiveness of selective breeding is constrained by the extended time required for fish to reach sexual maturity.

To achieve sexual maturity, fish must undergo gametogenesis, a process that leads to the production of sperm or ova in the gonads. This process is tightly regulated by a complex interplay of hormones and proteins released as the organism grows and develops. Primordial germ cells, which are present early in an organism's development, first increase in number before acquiring stemness, after which some differentiate into mature sperm or oocytes through multiple rounds of cellular division. The duration of this process varies depending on the species of fish. In males, spermatogonia are stem cells that can differentiate into spermatocytes, which then produce spermatozoa, commonly known as sperm (Suede et al., 2023).

Previous studies have successfully developed culture systems that allow for the complete process of spermatogenesis to occur in vitro (Sakai, 2006; Kawasaki et al., 2015). Additionally, it has been demonstrated that sperm from *Gnathopogon caerulescens* can be obtained from cultures derived from six-month-old fish maintained for only six weeks (Higaki et al., 2017). This species typically takes a year to reach sexual maturity; thus, if this method can be adapted for other species, the time required for another generation of selective breeding could be halved. Such an innovation could significantly reduce the time between generations, offering aquaculturists a substantial advantage if the necessary conditions for other fish species

are discovered. Currently, this technique is applicable to only a limited number of species. By establishing the conditions needed to culture *Danio rerio* spermatogonia and facilitate their differentiation into spermatozoa, this paper aims to lay the groundwork for researchers to identify the conditions required for other species.

Methods

Animal Husbandry

Danio rerio used in this study were between 3 and 12 months old. They were housed under standard conditions at the National Institute of Genetics in Mishima, Japan. Adults (49 days old and up) were kept in a circulatory system with the temperature maintained at 28°C. They were fed twice daily with a combination of live brine shrimp and generic adult fish food.

Juveniles were divided into two groups to test whether adding powdered food at various stages of development would enhance their overall growth. Both groups were fed rotifers from 5 days post-fertilization (dpf) until 9 dpf. After 9 dpf, rotifers were replaced with brine shrimp. The control group was only given live food. The test group received various powdered foods as they matured, with 75, 150, and 300-micron powders used until the fish reached adulthood.

Enriched L-15 Media for Culture

To culture spermatogonia, a layer of supporting feeder cells must first be established. The basic testicular cell culture medium, based on Sakai (2006), must be prepared fresh before starting the culture. L-15 medium was enriched with Milli-Q water, 10% BSA, Penicillin/Streptomycin, Kanamycin, L-Glutamine, Hepes, $CaCl_2$, FBS, Gonadotropin, 2-mercaptoethanol, Heparin, and *Danio rerio*-specific embryonic extract. The concentration of each component was adjusted based on the number of embryos in the embryonic extract. Once

the medium is prepared, Sertoli cells can be cultured. Media changes are required every 2-3 days.

Replating Sertoli cells

Once the cells reach 60-70% confluence, aspirate the culture media and wash the cells with sterile PBS. After aspirating the PBS, add Accutase to the cells. The timing of Accutase treatment is crucial; if the Accutase is removed too soon, the cells will not detach, but prolonged exposure can lead to cell death. After the appropriate Accutase treatment, add fresh culture media to the cells. Using a sterile pipette, gently aspirate and release the media to help detach the cells, taking care to avoid creating bubbles, as cells can become trapped in them. Transfer the media containing the detached cells into new cell culture dishes and incubate at 28°C in air.

Embryonic extract

Using fewer than 1,000 *Danio rerio* embryos at 3 days post-fertilization (dpf), begin by removing the chorions. Transfer all embryos to a large dish and euthanize them on ice. Move the embryos to a 100 ml beaker on ice, and add 100 ml of 0.5% bleach (NaClO) in chilled Milli-Q water. Decant the bleach solution and add 100 ml of Fish Ringer Solution. Incubate for 2 minutes, repeating this wash twice. Transfer the embryos to a 15 ml dounce homogenizer, removing most of the liquid. Homogenize the embryos five times, then add 10 ml of L-15 medium. Continue homogenizing 30 more times, and then transfer the mixture into two 15 ml conical tubes.

Testes dissection and Collagenase treatment

Danio rerio are euthanized by immersion in 0.01% ethyl p-aminobenzoate. The testes are removed from juvenile or adult *Danio rerio* and soaked in L-15 medium. Four to five sets of testes are collected to ensure sufficient tissue. The testes are then transferred into PBS containing 0.05% bleach (NaClO) and immediately moved to a clean dish with L-15 medium.

The testes are minced and added to a solution of 500 µ/ml collagenase broth for 3 hours. The conical tube containing the collagenase and minced testes is placed in a shaking water bath at 26°C.

Cell Culture Spermatogonia

The night before starting the culture of spermatogonia, treat Sertoli cells with mitomycin C to arrest their growth. Add 20 µ/mL of mitomycin C and L-15 medium to the Sertoli cells and incubate for 3 hours at 28°C. Wash the cells three times with PBS, allowing 2-5 minute intervals between washes. Replate the cells using Accutase into a clean petri dish. Once the collagenase treatment is complete, add 7 ml of L-15 medium to the conical tube containing the collagenase and minced testes. Centrifuge the tube at 800 rpm for 10 minutes at 26°C. Remove most of the supernatant and resuspend the cells in the culture medium. Add the culture medium to the feeder cells and incubate at 28°C.

In vitro fertilization

This protocol follows the methods outlined by Sakai (2006) for in vitro fertilization. After 2-3 days sperm may be collected from cultured spermatogonia and spermatocytes. The day before sperm collection, replace half of the culture media. On the day of collection, gently pipette the media up and down to bring the sperm into suspension. Remove the media and transfer into a 15 ml conical tube. Centrifuge at 800 rpm for 10 minutes, then remove most of the supernatant and resuspend the sperm in the remaining media.

To prepare the oocytes, separate female *Danio rerio* the night before. In the morning, anesthetize the female *Danio rerio* in 0.01% ethyl p-aminobenzoate until there is no response to touch. Remove the fish from the anesthesia and place it on its side in a glass petri dish. Gently stroke the female's abdomen with a slightly wet finger to encourage egg release. Continue stroking until all eggs are released; if no eggs appear after approximately five strokes, select a different female. Add the sperm solution directly onto the oocytes and gently shake the dish for

two minutes. Place the dish in an incubator at 26°C for 5-6 hours, then remove any unfertilized eggs.

Results

The modification to the *Danio rerio* diet did not yield the expected results. For the initial two weeks, the control group was larger than the test group and appeared approximately one week older. However, after the two-week mark, the test group quickly caught up and surpassed the control group in size.

Figure 1A displays confluent Sertoli cells maintained in culture for 3 days before being treated with mitomycin C to arrest growth and development. Figure 1B shows another example of confluent Sertoli cells that had also been cultured for 3 days. Only confluent cells were suitable for proceeding with the testicular cell culture.

Figure 2A illustrates successful spermatozoa culture, with the white arrows indicating the sperm. Although only the heads of the sperm are visible, their movement can be tracked. Figure 2B depicts the successful culture of spermatogonia. In Figure 2C, in vitro fertilization (IVF) is demonstrated by the successful creation of embryos.

Figure 3A shows the removal of all chorions and the placement of embryos into a large petri dish on ice for euthanasia preparation. Figure 3B depicts the homogenization of embryos in L-15 media.

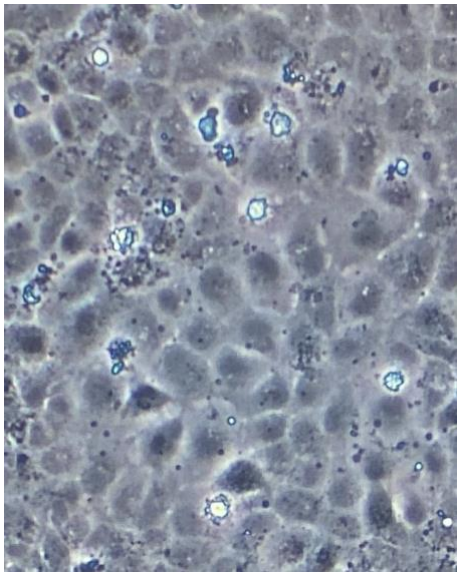

Figure 1A: Confluent Sertoli Cells in culture.

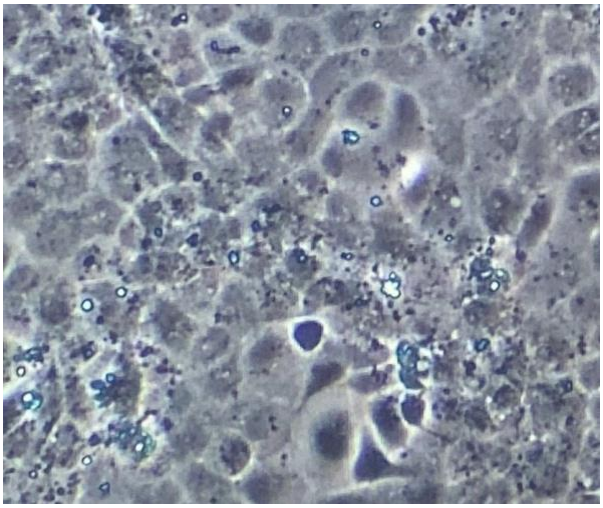

Figure 1B: Another example of confluent Sertoli Cells in culture.

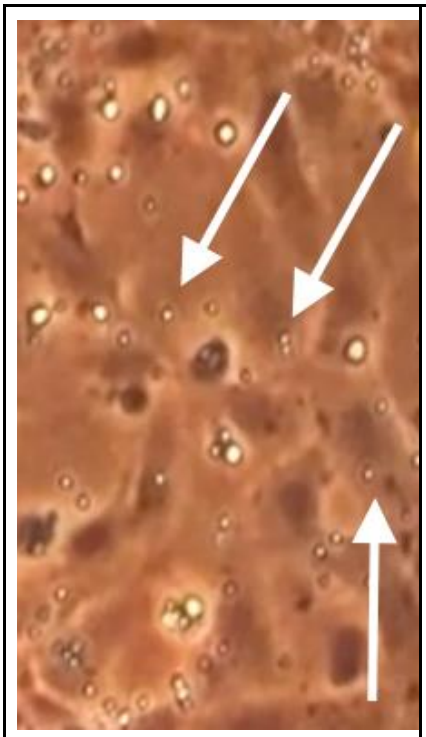

Figure 2A: Testicular cells cultured in media. Smaller white circles are pointing to the spermatozoa.

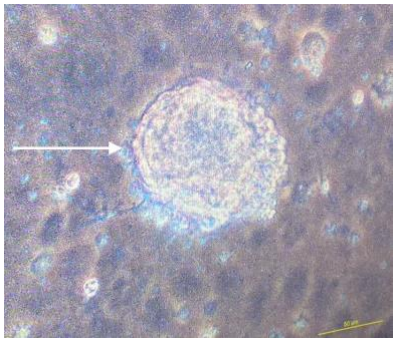

Figure 2B: Testicular cells cultured in media. The white arrow is pointing to spermatogonia.

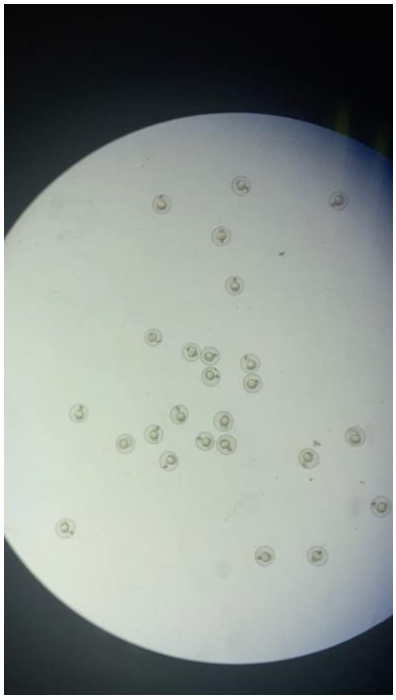

Figure 2C: Healthy embryos produced via IVF.

Figure 3A: <1000 *Danio rerio* embryos with removed chorions combined and placed on ice.

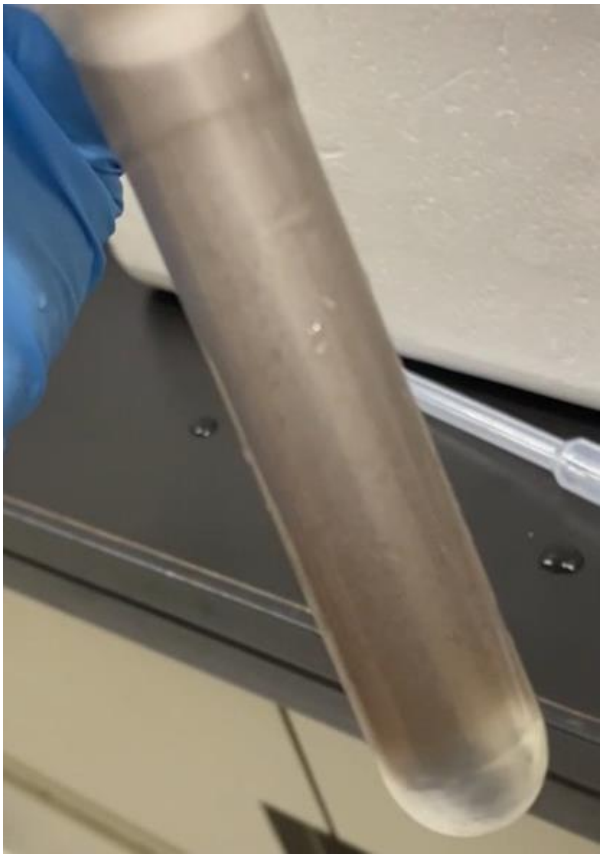

Figure 3B: A dounce homogenizer containing <1000 *Danio rerio* embryos combined with L-15.

Discussion

Animal husbandry is crucial for ensuring the proper care of animal specimens. In a two-month growth study, embryonic *Danio rerio* from the same parents were divided into two equal test groups. The aim of this study was to identify optimal feeding conditions to promote rapid growth and adequate nutrition among the test subjects. The control group was fed a live food diet throughout its lifespan, which included rotifers from 3 days post-fertilization (dpf) to 9 dpf, followed by brine shrimp. The test group received the same live food diet but was also supplemented with 75-micron powdered food from 6 dpf to 12 dpf, 150-micron powdered food from 13 dpf to 21 dpf, a mixture of 150/300-micron food until 60 dpf, and finally, 300-micron food

mixed with adult food from 60 dpf to 90 dpf. In addition to the feed differences, the control group was kept in a large petri dish until 15 dpf. After this point the control group was transferred to an adult sized tank with water circulation. Meanwhile, the test group was transferred into an adult-sized tank at 6 dpf with a low water level and no water flow at first. The water level was gradually increased and at 12 dpf circulation of the water was enabled and automated by the system.

For the first two weeks, the *Danio rerio* in the control group were significantly larger. However, after this point and for the remainder of the experiment, the group receiving the supplemental powdered food grew to adulthood more quickly and maintained a larger size. I believe the initial size advantage of the control group was due to the longer time the embryos and juveniles remained in the large petri dish before being moved to the system tanks. In a more confined space, they did not have to exert as much effort to find food. However, once the test group reached a certain size and began consuming the powdered food, they quickly caught up and surpassed the control group. To minimize variables and accurately assess the impact of powdered food on growth, it is essential to ensure consistent housing conditions for both test groups in future experiments.

This report details the successful development of a spermatogonial culture system capable of producing functional sperm, which was subsequently used to create embryos through artificial fertilization. The successful culturing of the Sertoli feeder cell layer took several weeks due to multiple instances of contamination. The feeder cell layer is crucial because these cells support the differentiation and division of other cells (Llames et al., 2015). Given that the goal was to culture underdeveloped testicular cells from younger males under conditions that promote differentiation, it was essential to establish a healthy culture of Sertoli cells as the feeder layer. However, contamination issues persisted throughout the experiment, delaying the culturing process. The increased humidity associated with Japan’s rainy season may have contributed to these problems.

To prevent further contamination, special care was taken to ensure proper decontamination of all objects entering the clean hood. All items, including the researcher's gloved hands, were thoroughly soaked in 70% ethanol. Additionally, all liquid media and culture dishes were kept upright to prevent any splashing onto the lids. Once the jostling of the culture dishes was minimized, contamination was no longer an issue. Further measures included cleaning the incubator to remove any fungal growth and disinfecting the larger dishes holding the culture dishes with 70% ethanol whenever they were removed from the incubator. Once contamination was under control, the splitting of Sertoli cells became successful. After reaching a high level of confluence, a mitomycin C treatment was applied to halt the division of the Sertoli cells, as is typical for feeder layers (Llames et al., 2015).

The following day, testes from five male *Danio rerio* were extracted and disinfected using 0.5% bleach in PBS. The testes were then transferred into L-15 medium to remove any residual bleach, minced in a collagenase solution, and incubated in a 28°C water bath for three hours. Every 20 minutes, the mixture was manually stirred to assist in tissue breakdown. After the treatment, 7 mL of L-15 medium was added to the sample, which was then centrifuged for 10 minutes at 800 rpm. Most of the supernatant was discarded, and 1.8 mL of culture media was used to resuspend the pellet. The cell culture media on the prepared Sertoli cells was then aspirated, and the culture media containing the testicular cells was transferred onto the Sertoli cells. The sample was incubated in air at 28°C.

Interestingly, no contamination was observed once the testicular cells were cultured. Given that the tissue was directly extracted from the animal, this lack of contamination was unexpected. Careful dissection techniques were employed to prevent any additional connective tissue or fecal matter from entering the PBS with the testes. The culture media was changed every three days, and the night before sperm extraction, half of the media was replaced. Sperm extraction was performed by carefully aspirating and dispersing the media across the dish to increase the concentration of sperm in the cell culture media. All of the media was then

transferred into a 15 mL conical tube and centrifuged at 800 rpm for 10 minutes. The supernatant was aspirated, leaving 100-200 µL, in which the pellet was resuspended. On the same day, oocytes from a female *Danio rerio* were obtained by gently stroking the abdomen. Sperm extraction was successful, as oocytes fertilized with the extracted sperm developed into embryos. Repeated experiments will be necessary to determine whether this method can be consistently reproduced. However, this culture system shows promise for *Danio rerio* testicular cells and may serve as a model for other fish species. If this system can be leveraged in other species of fish, just like with the *Gnathopogon caerulescens,* time to reach sexual maturity can be halved. This could help aquaculturists move forward with selective breeding much faster than average which can lead to better crops of fish for all to enjoy.

Acknowledgements

I would like to express my deepest gratitude to Dr. Noriyoshi Sakai and his exceptional team for their unwavering guidance and encouragement. Dr. Sakai's mentorship challenged me to grow in a nurturing environment. I am particularly thankful to Dr. Yukiko Imai and Fabien Velilla, whose patience and assistance were crucial to the success of this project. To the rest of Dr. Sakai’s team, I appreciate all your help.

I would also like to thank the NSF for funding this program, which has allowed me to develop both as a researcher and as a global citizen in Japan. Special thanks to Dr. Mika Munakata and Dr. Carlos Molina for their continuous guidance throughout my journey in Japan.

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