JOÃO GIL GOMES FERREIRA

DEVELOPMENT OF 3D CELL CULTURES AND TESTICULAR ORGANOID TRANSPLANTATIONS: POTENTIAL TOOLS FOR REPRODUCTIVE BIOLOGY

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Mestrado em aquacultura e pescas

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Trabalho efetuado sob orientação de:

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Declaration of authorship of work

I declare I am the author of this work, which is original and unpublished. The sources consulted have been duly cited in the text and included in the list of references.

Acknowledgement

I want to take this opportunity to acknowledge all the support given to me during this final phase of my master's degree.

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Abstract

Researchers in the last decade have struggled to increase the number of offspring of species with economic importance or endangered status, reducing aquaculture growth. The primary obstacles are spermatozoa's low number and motility and the long maturation period in some fish species. Germ cell transplantation and cryopreservation techniques enable researchers to surpass problems related to the quality and quantity of spermatozoa. However, the most significant bottlenecks are the low number of spermatogonia germ cells and the incapability to cryopreserve oocytes. Organoids are cell structures only produced in 3D cell mediums, capable of maintaining functionality and structure similar to ***in vivo.*** Cell proliferation and differentiation in organoids is the most critical part of their development and an essential step in transplanting them into recipients since there is a probability of increasing the colonisation of the gonadal tissue and decreasing the gonadal maturation period. Therefore, the study and usage of organoids to study epigenetics, cell-cell communication, and cell differentiation are recent strategies in fish biology. The current project aims to reach a consensus on the definition of organoids and be the steppingstone for future work done with fish organoids. Two protocols were produced during these experiments, one for organoid development utilising vitrogel and a second for organoid development through aggrewell plates. The aggrewell procedure was more efficient than any vitrogel, producing more organoids during a smaller period. Despite both mediums achieving organoid-like structures, histology proved that only the aggrewell successfully created organoids with a single membrane and niche structure similar to the donor organ. Preliminary tests with transplanted organoids showed positive results, with observable organoids after 45 days. In summary, the performed experiments will facilitate any work related to future works with fish organoids, surpassing any difficulties in organoid development.

Keywords: Aquaculture, Sturgeon, Cell culture, Organoid, Transplantation

Resumo

Durante as últimas décadas, várias publicações surgiram com o intuito de melhor os processos reprodutivos de animais aquáticos, procedimentos que introduziram a criopreservação, micro-trasplantes e cultura celular de células germinativas. Porem os investigadores têm se deparado com dificuldades em descobrir novos métodos capazes de permitir o aumento do número de descendestes em espécies com valor económico e em estado de perigo de extinção, reduzindo o crescimento da aquacultura. Os principais obstáculos são, o reduzido número de espermatozoa e a sua reduzida mobilidade, a ineficácia na criopreservação de oócitos e os longos períodos de maturação de algumas espécies. Porem o número reduzido de espermatogónias e a incapacidade de crio-preservar oócitos representam as maiores dificuldades do momento. Devido as presentes dificuldades diversas grupos científicos deslocaram o seu foco para o processo reprodutivo, o desenvolvimento das células e a sua diferenciação. Através da utilização de culturas celulares de células germinativas já existentes e subtis alterações dos meios de cultivo (capazes de manter uma estrutura tridimensional) e a adição de hormonas fundamentais no processo de organização e diferenciação celular, foi possível observar uma reorganização das células no meio e a criação de estruturas denominadas esferoides, e eventualmente o desenvolvimento das mesmas e a chegada ao termo organoide. Organoides são estruturas de celulares produzidas em meios de cultura tridimensionais, que são capazes de manter a funcionalidade e a estrutura semelhantes ao órgão *in vivo*. A comunicação celular em organoides representa a parte mais critica do seu desenvolvimento, uma vez que durante o processo de formação dos organoides as células têm que através de comunicação célula-célula proceder à agregação, proliferação, diferenciação e reorganização de modo a obter uma estrutura semelhante à do órgão dador (membrana celular visível e reorganização celular semelhante ao nicho de onde foram retiradas). O estudo e uso dos organoides em epigenética, comunicação celular, e diferenciação representam uma nova estratégia no estudo da biologia reprodutiva em peixes. O projeto atual visa chegar a um consenso sobre a definição de organoides em peixes uma vez que ainda existem incoerências em vários artigos científicos, (existindo diversos artigos que consideram organoides esferoides e outros que consideram esferoide como sendo organoides) bem como ser o catalisador para trabalhos futuros realizados com organoides em peixes. Durante o período experimental, dois protocolos foram produzidos, um para o desenvolvimento de organoides utilizando diferentes vitrogeis (utiliza uma matriz tridimensional artificial e flexível semelhante a *in vivo* que permite a movimentação das células, a sua agregação em estruturas 3D) com o intuito de distinguir o mais favorável às células utilizadas, e um segundo com o objetivo de desenvolver organoides através de placas aggrewell (que utilizam força centrífuga e uma placa especifica para aggrewell permitindo assim agregar as células de forma homogenia e obtendo estruturas semelhantes ao longo de toda a placa. Após o procedimento experimental estar concluído, foi possível observar que o protocolo de aggrewell foi o mais eficiente (devido ao método utilizado e à placa, foram observados 1200 organoides com uma media de tamanho de 130.96µm), ultrapassando os resultados obtidos pelo vitrogel 1 (o único vitrogel capaz de produzir organoides).entre os vitrogeis o vitrogel 2 e 4 foram capazes de formar estruturas esferoides mas sem reorganização interna do organoide, por outro lado no vitrogel 3 as células demonstraram dificuldades em se deslocarem e por consequência tiverem dificuldades na fase de agregação. Apesar de não existirem diferenças estatísticas relativamente ao tamanho o mesmo não foi observado relativamente ao tempo necessário para a sua produção, (o protocolo de aggreweel necessita de uma duração de 7 dias e o vitrogel de uma duração de 21) e o número obtido (o aggrewell obteve em média 1200 organoides e o vitrogel uma media de 6). O aggrewell produziu mais organoides durante um menor período de tempo. Apesar de ambos os meios alcançarem estruturas semelhantes a organoides, as análises histológicas realizadas demostraram que apenas os organoides obtidos através do aggrewell tiveram sucesso na produção de organoides, sendo estes os únicos capazes de obter organoides com membrana única visível e estrutura de nicho semelhante ao órgão dador (estruturação das células de Leydig, células de Sertoli e espermatogónios). Durante as análises histológicas é necessário salientar que não foi possível distinguir espermatozoa no interior dos organoides. Com o intuito de testar a qualidade dos organoides produzidos, e a hipótese de a estrutura multicelular dos mesmos lhes conferir uma maior resiliência contra a pressão do elemento recetor pós-transplante (comparar a taxa de sobrevivência dos organoides pós-transplante com a taxa de sobrevivência de células individuais pós-transplante), foram realizados testes preliminares. Os organoides produzidos pelo protocolo de aggrewell (uma vez que estes representam a melhor aproximação a um sistema *in vivo*) foram transplantados para larvas estéreis de esturjão 15 dias após a sua eclosão. Os organoides foram corados utilizando PKH-26 (Sigma Aldrich) responsável por corar de vermelho a membrana lipídica das células. As larvas foram anestesiadas utilizando 0.03% 2-phenoxyethanol e os organoides foram micro-transplantados para uma zona especifica da larva, entre o intestino e a bexiga natatória. A transplantação foi confirmada através da visualização de organoides corados através de uma lupa de florescência no interior das larvas. Duas observações foram realizadas, a primeira observação 21 dias pós eclosão e a segunda 45 dias pós eclosão. Um total de 10 larvas foram sacrificadas por observação utilizando 0.03% 2-phenoxyethanol como elemento eutanaziante. Ambas as observações realizadas mostraram larvas transplantadas com sucesso, sendo visível células marcadas com PKH-26. Ao dia 21 foi possível observar que 9 em 10 larvas mostraram sinais de marcação, mas sem aparente proliferação colonização dos tecidos. No dia 45 apenas 5 das 10 larvas sacrificadas demostravam marcação porem não foi possível visualizar qualquer proliferação. Em resumo, os protocolos criados durante esta tese tem o intuito de aproximar qualquer investigador interessado em iniciar o seu percurso em organoides testiculares de peixe (principalmente em organoides testiculares de esturjão) da informação mais recente relativamente ao tema, evitando a repetição de experiencias (relacionadas com a mesma espécie) e proporcionando dados que permitem servir de fundação a novos testes e experiencias, superando assim quais queres dificuldades no desenvolvimento de organoides.

Palavras-chave: Aquacultura, Esturjão, Culturas Celulares, Organoides, Transplantes

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1. Introduction

Understanding fish reproductive biology is critically important for aquaculture and as a means of conservation and protection, enabling the development of artificial reproductive methods and increased efficiency of reproductive aquaculture programs. Despite all the effort put into artificial reproduction, most of the success has proved to be limited to captive broodstock (Ghiasi et al., 2017). With the growing need for optimised ways of increasing the number of offspring of species with either economic importance or endangered status, germ cell transplantation can be an essential tool to preserve precious genetic resources (Iwasaki-Takahashi et al., 2020).

1.1. Germ cell transplantation

Developing germ cell transplantation techniques is a significant breakthrough in surrogate germ cell production and chimaera. Pioneering studies involved transferring primordial germ cells (PGCs) to the bloodstream of developing chicken embryos, resulting in germline chimaeras and donor-derived offspring (Tajima et al., 1993). Similar techniques were later established in rodents by transplanting spermatogonial stem cells (SSCs) from a donor species into the seminiferous tubules of an infertile recipient, allowing for the reestablishment of spermatogenesis (Brinster & Zimmermann, 1994). These techniques have not only expanded our understanding of SSC physiology but also had significant applications in experimental animal research and zootechnical science, preservation of endangered species, and production of transgenic animals (Dobrinski., 2008; Hill & Dobrinski, 2006; Kanatsu-Shinohara et al., 2008; McLean, 2005; Orwig, 2005). Although germ cell transplantation is well established in mammals, it has only recently been applied to fish species with successful transplantation of trout primordial germ cells or spermatogonia into newly hatched salmon or trout embryos resulting in donor germ cell development (Figure 1.1), (Meistrich, 1993).

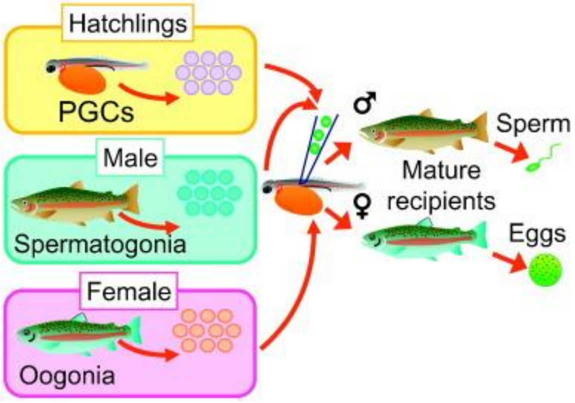


Figure 1.1 Primordial germ cell, spermatogonia, oogonia transplantation to a recipient and production of donor-derived gametes (Yoshizaki & Lee, 2018).

Researchers have successfully performed germ cell transplantation in fish using trout primordial germ cells (Takeuchi et al., 2004) or spermatogonia (Okutsu et al., 2006, 2007), which were injected into newly hatched salmon or trout embryos. These transplanted germ cells could migrate into the undifferentiated gonad and develop into male or female gametes depending on the recipient's sexual fate. A germ cell transplantation technique for Siberian sturgeon was also established using sterlet larvae as recipients, but the low number of SSCs limits its effectiveness. According to Meistrich, in 1993, there were only about two SSC per 104 testicular cells in mice, which make up only 0.03% of total germ cells (Tagelenbosch & de Rooij, 1993).

1.2. Male Germ Cells

Germ cells are but a steppingstone in the differentiation process of primordial germ cells (PGC), shown to be present *“in vivo”* as a small population of cells that initially separated from other cell lineages in the embryonic life of most animal species (Wylie, 1999). Environmental cues can signal germ cell differentiation into gametes of either sex (Wylie, 1999). Young animals will contain more type A undifferentiated spermatogonia, differentiating type A1–A4 spermatogonia, and probably not more than about 1% of stem cells (Griswold et al., 1989; van Pelt et al., 1995; van Pelt & de Rooij, 1990).

In fishes, a specific type of undifferentiated spermatogonia called Aund, plays a vital role in the development of germ cells. These Aund spermatogonia maintain the unique ability to self-renew, differentiate, and transform into various germ cell types (Schulz et al., 2010). They are also the biggest germ cell in the fish testis and are present in a small portion of the type A spermatogonia population (Xie et al., 2020). However, to perform germ cell in vitro culture, cryopreservation, and transplantation, a highly purified population of type Aund spermatogonia is required. Unfortunately, the proportion of Aund spermatogonia is low in the testes compared to other germ cell types (Schulz et al., 2005). To overcome this challenge, flow cytometric cell sorting (FCM) and fluorescence-activated cell sorting (FACS) are two precise and efficient methods to obtain pure and homogenous cell culture populations. These methods separate cells based on size, shape, granularity, self-fluorescence properties, or specific surface antibodies conjugated with fluorescence dyes (Xie et al., 2020).

1.3. Cell Culture system 2D vs 3D

The two-dimensional model is the most commonly used cell culture system, but lately, the three-dimensional culture system has gained popularity (Pampaloni et al., 2007). The behaviour of cells varies depending on the type of cell culture system used (Sanyal, 2014). In adherent two-dimensional (2D) cultures, cells grow as monolayers in a culture flask or flat petri dish (Breslin & O'Driscoll, 2013). The advantages of 2D cultures are that they are easy and cost-effective to maintain. However, adherent 2D cultures also have several drawbacks. Firstly, they do not replicate the natural structures of tissues or tumours. In this culture method, cell-cell and cell-extracellular interactions are not represented as in the tumour mass. These interactions are responsible for cell differentiation, proliferation, gene and protein expression, drug metabolism, and other cellular functions (Baker & Chen, 2012; Bissell et al., 2003; Pampaloni et al., 2007). After isolation from the tissue and transfer to 2D conditions, the morphology of the cells changes (Figure 1.2), as well as cell division. The 2D culture system also results in the loss of diverse phenotypes (Petersen et al., 1992; von der Mark et al., 1977), which can affect their function (Kilian et al., 2010; Mahmud et al., 2009), the organisation of the structures inside the cell, secretion, and cell signalling (Debnath & Brugge, 2005; Nelson & Bissell, 2006). Due to the disruption of interactions with the external environment, cells growing adherently lose their polarity (Masaka T et al., 2007), which can alter the cellular response to various stimuli and induce apoptosis (Meyers et al., 2006; Weaver et al., 2002). Another disadvantage of the 2D culture system is that the cells in the monolayer have unlimited access to the medium's ingredients, including oxygen, nutrients, metabolites, and signal molecules, unlike in vivo. Due to the many disadvantages of 2D systems, alternative models that better replicate the natural tissue environment, such as 3D cell culture systems, could be more realistic and similar to in vivo conditions.

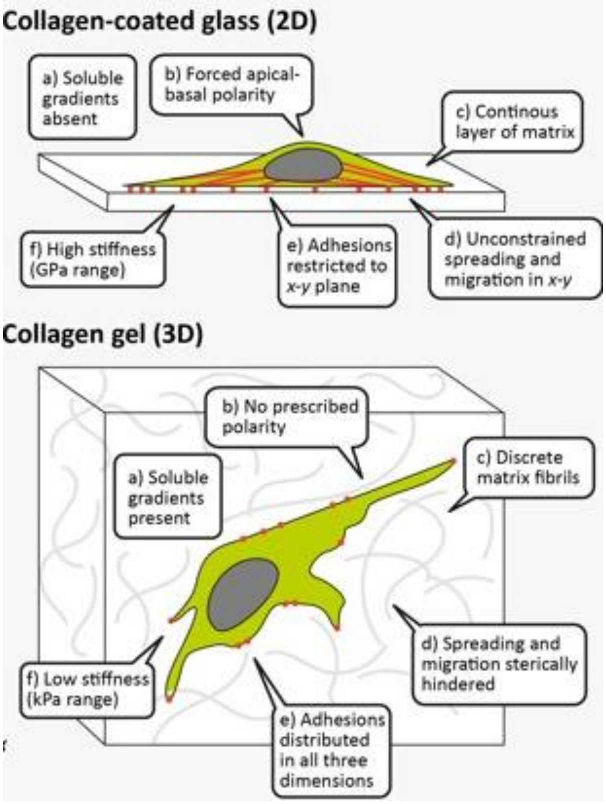


Figure 1.2 Microenvironments structure alters cell morphology (Baker & Chen, 2012).

1.4. Organic scaffold vs. synthetic scaffold

Many research groups have explored scaffold-based culture methods to recreate a 3D microenvironment to facilitate cell-cell and cell-environment interactions, nutrient access, and cellular mechanisms (Cham et al., 2021). Scaffolds provide mechanical properties crucial for cell behaviour (adhesion, migration, proliferation, differentiation). Generally, organic or natural scaffolds (Figure1.3A) are formed by biological materials, such as collagen, laminin, and entactin, that provide biocompatible, biodegradable, and low cytotoxicity. Unfortunately, this type of scaffold suffers rapid degradation, showing variable composition depending on the source, and sometimes their composition is undefined. On the other hand, synthetic scaffolds (Figure1.3B) are artificially constructed polymers with well-defined components (ideal for bioprinting), low biocompatibility and lack of cell adhesion molecules.

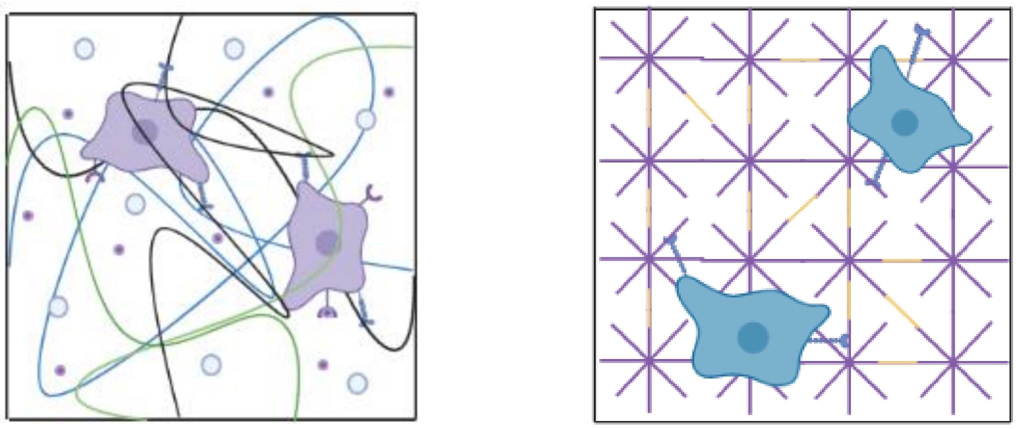


Figure 1.3 Organic medium-(A)-Black line (Collagen IV); Green line (Entactin); Blue line (Laminin); Blue ball (Xenogenic contaminant); Blue receptor (Integrin); purple receptor (GF receptor) Purple cell (Stem cell); Synthetic medium-(B)- Blue cell (Stem Cell); Blue receptor (Integrin); yellow line (non-degradable or degradable crosslinker); purple lines (Polymer matrix).

1.5. VitroGel®Organoid

Vitrogel is an artificial hydrogel that replicates donor cell tissue's natural structures and stiffness with remarkable accuracy. It is highly effective in supporting the development of organoids, with four different types of hydrogels available under the brand, each designed to meet specific conditions for organoids. The hydrogels are xeno-free, transparent, and permeable, making them compatible with various imaging systems. The 3D matrix formation of the hydrogel is incredibly accessible, involving simply mixing the hydrogel solution with a cell-culturing medium (Figure1.4). Overall, Vitrogel is a truly remarkable technology that has the potential to revolutionise the field of organoid development.

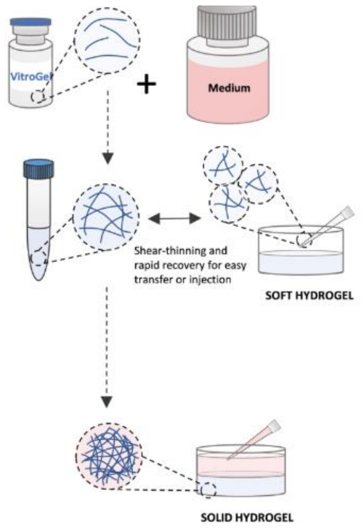


Figure 1.4 3D encapsulation method of Vitrogel (TheWell Bioscience Inc.).

1.6. Spheroids vs Organoids

3D models support cell-cell and cell-environment interactions similar to in vivo systems, allowing for the maintenance of stimuli, cell morphology and polarity (Kapałczyńska et al., 2016). These *in vivo-like* models enable the cells to form spheroid structures (Figure1.5 A), agglomerates of cells that do not maintain either the architecture of the original tissue or its functionality, and organoids (Figure1.5 B) which are more complex, capable of maintaining both architecture and function of the original tissue (resemble fragments of organs) (Sakalem et al., 2021). Several different organs, such as the intestine (Sato et al., 2009), stomach (Barker et al., 2010), pancreas (Huch et al., 2013), liver (Takebe et al., 2013), vasculature (Morgan et al., 2013), colon (Sato et al., 2011), pancreas (Boj et al., 2015), mammary gland (Simian et al., 2001)and brain (Quadrato et al., 2017) presented successful results in 3D organoid systems.

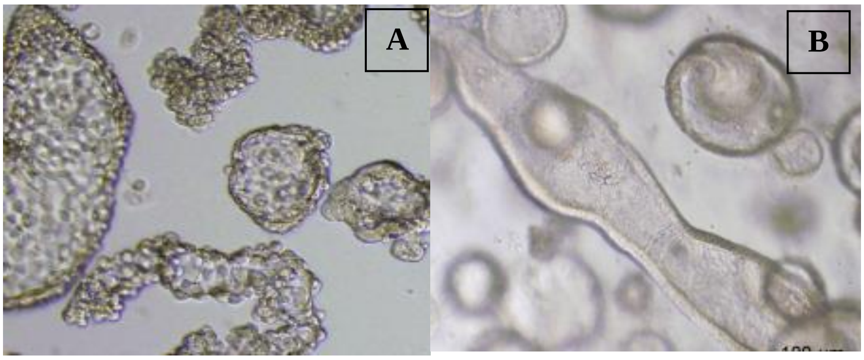


Figure 1.5 (A)-Spheroid; (B)-Organoid (Bourdon et al., 2021).

1.7. Testicular organoids

Despite the increased interest in organoid systems over the last decade, it is only very recently that testicular organoids have started to gain interest (Alves-Lopes et al., 2017; Baert et al., 2017; Pendergraft et al., 2017; Sakib, Voigt, et al., 2019; Strange et al., 2018). Baert and collaborators (2017) were pioneers in the utilisation of testicular organoids of pre-pubertal humans that, despite lacking the organisation of testis, were still capable of producing testosterone, inhibin B, cytokines and the Sertoli cells were able to form tight junctions (Baert et al., 2017). These organoid models can contribute to further studies on spermatogenesis and investigate the influence of toxic agents in the testis (Nóbrega R personal communication). Dr. Ina Dobrinski recently developed a technique capable of producing testicular organoids using microwells and centrifugal aggregation. The generated organoids were spherical and had both Leydig and endothelial cells in the interstitial compartment; meanwhile, the external surface was formed by Sertoli cells and germinative cells (Sakib, Uchida, et al., 2019).

Regardless of the studies done in mammals, up to this date, no studies have been done in the testicular organoids of fish. The lack of knowledge in this area of expertise represents a handicap in what could be a more realistic way of studying and understanding spermatogenesis, proliferation, and differentiation of testicular cells.

1.8. Fish Model: Sturgeon

Sturgeon (Chondrostei, Acipenseriformes) is known as "ancient Actinopterygian", valued for their meat and caviar (Bemis et al., 1997). They inhabit the northern hemisphere's rivers, estuaries and inland seas and reproduce in freshwater (Bemis & Kynard, 1997). Their evolutionary history dates to about 200–250 million years. In the last century, most natural stocks inhabited the basins of the Sea of Azov, the Black Sea, and the Caspian Sea (90% of the world's population) (Inoue et al., 2005). Since the beginning of the 21st century, almost all 27 sturgeon species from Acipenseridae and Polyodontidae are threatened or endangered, and their natural stocks are decreasing dramatically due to overfishing and environmental degradation, including changes affecting migration and reproduction (Alavi et al., 2012; Bronzi & Rosenthal, 2014). Over-exploitation of natural and enhanced sturgeon stocks for caviar production and severe habitat deterioration has led to drastic declines in natural populations. Consequently, IUCN in 1997 listed all commercially utilised sturgeon species worldwide in Annex II of the CITES regulations, thus requiring worldwide agreed quotas (Vasilyeva et al., 2019) for trade to promote protection (Bronzi & Rosenthal, 2014). Due to this dramatic decline in the number of natural sturgeon populations, sturgeon aquaculture has emerged, particularly in the states located within the areas where sturgeons are naturally prevalent (Vasilyeva et al., 2019).

With the growing need for optimised ways of increasing the number of offspring of species with economic importance and with an endangered status, researchers have turned their studies towards germ cell transplantation, germ cell in vitro culture and cryopreservation as means of preserving precious genetic resources of endangered fish species (Iwasaki-Takahashi et al., 2020) (Figure 1.6).

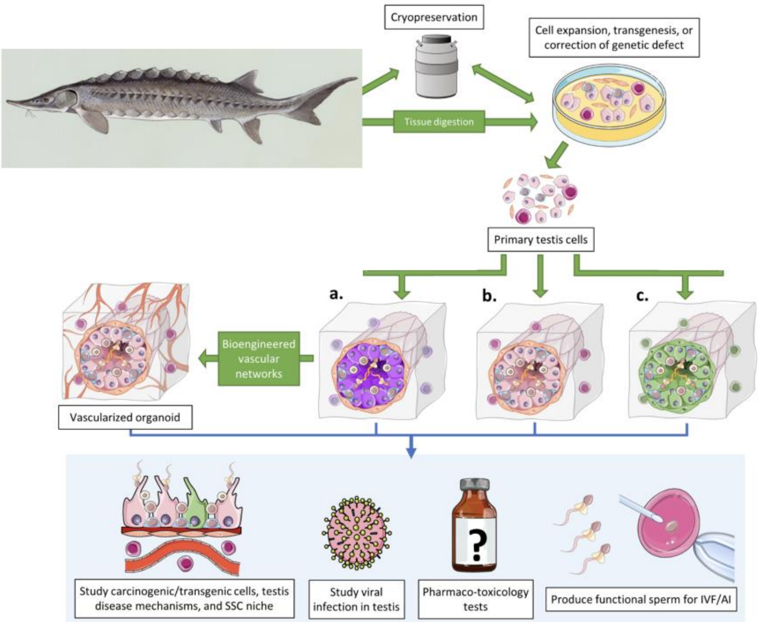


Figure 1.6 Future perspectives of scaffold-based testis organoids for fish, modified from Cham et al., (2021).

1.9. Thesis Objective

This thesis aims to enhance aquaculture tools by advancing 3D cell culture and exploring the feasibility of organoid transplantations in the future. Two distinct protocols for organoid formation will be created, and their efficiency will be compared based on cell performance and organoid formation. Additionally, the thesis will focus on characterising the cells and structure within the organoid to validate it is in vitro formation.

2. Materials and Methods

2.1. Ethics

Experiments performed in the Czech Republic followed the Animal Research Committee of the Faculty of Fisheries and Protection of Waters (FFPW), University of South Bohemia, Czech Republic. Fish were maintained according to the principles based on the EU harmonised animal welfare act of the Czech Republic principles of animal care in compliance with the national law (Act No. 246/1992 on protecting animals against cruelty).

2.1.1. Fish selection

Specimens of sterlet *Acipenser ruthenus* (1-2 years old) and larvae were reared in the hatchery facility of the FFPW. Sterile sturgeon larvae were used as recipients of testicular organoids.

2.1.2. Testicular tissue sampling

Specimens were euthanised through the severing of the spinal cord, and a section of the caudal fin was collected and stored in 100% ethanol for genotyping. Before dissection, fish were thoroughly washed with water (to remove the excess mucus) and then rinsed with 70% ethanol (disinfection). All the procedures were carried out in a biosafety cabinet using sterile utensils for dissection. To excise the testes, the skin and underlying muscle on the ventral part of the fish, between the pectoral fins, were snipped. The skin and underlying muscle were cut along the ventral part until the anal fin to expose the internal organs. As the testes are located above the gastrointestinal tract on both sides of the swimming bladder (Figure 2.1 A), a section in the anterior part of the gastrointestinal tract was made, enabling the partial removal of the intestine without risking contamination and subsequently, the removal of the testis (the rear part of the testis is not used due to the physiological connection with the intestine). After the testes were septically excised, they were washed in PBS (Figure 2.1 B) with antibiotics (ampicillin, penicillin and streptomycin) at 10mg/ml (Sigma Aldrich) for 2 minutes, sterilised in 0,05% bleach (2 minutes), and then washed again in PBS with antibiotics for two more minutes.

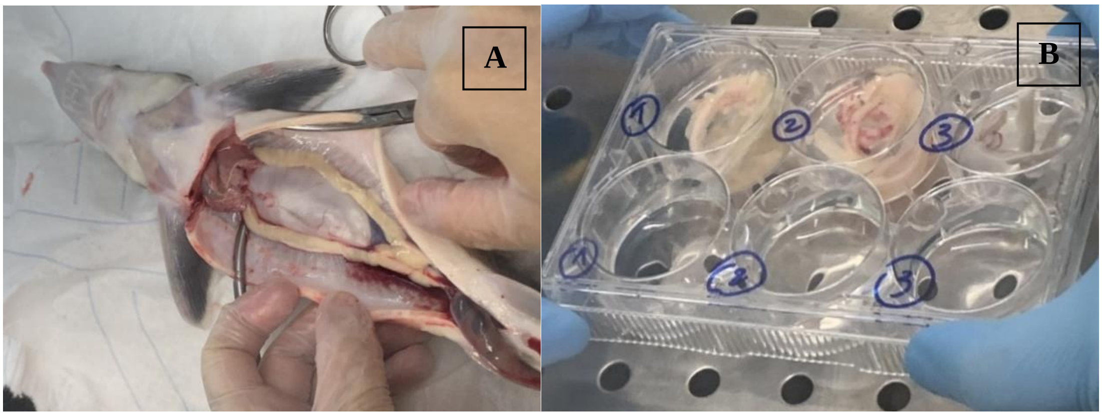


Figure 2.1 Dissection of the fish and gonadal removal (A), and subsequently rinse in PBS and 0.05% bleach (B).

2.1.3. Tissue dissociation and cell concentration assessment

The testicular tissue was dissociated through mechanical (Figure 2.2 A) and enzymatic methods (Figure.2.2 B). First, the gonads were minced into small pieces and centrifuged (100G for 5 minutes, 21ºC) to separate the fat from the gonadal tissue. The lipidic content and the supernatant were removed, and the tissue pellet was resuspended in an enzymatic dissociation medium (Annex 1.1 Protocol**)**.

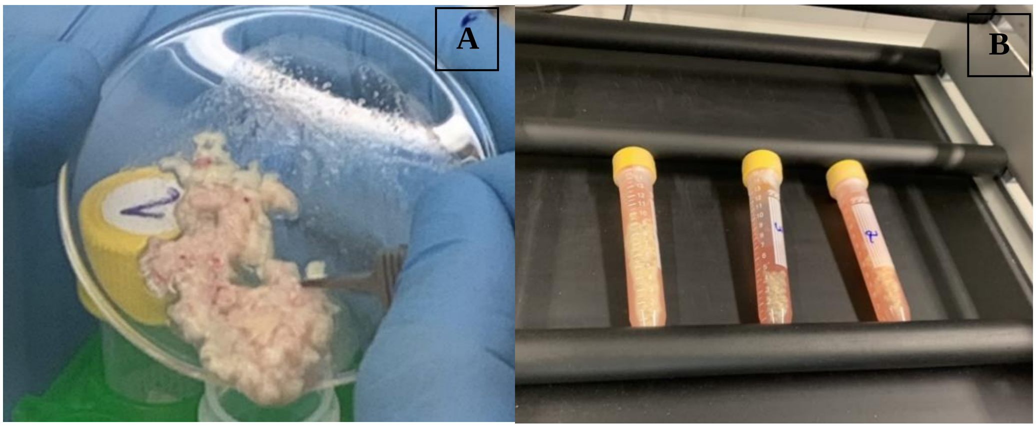


Figure 2.2 Mechanical dissociation of the gonads (A); Incubation of gonadal tissue in dissociation medium under shaking (100rpm) (B).

The enzymatic dissociation medium is a solution of L-15 medium containing different enzymes such as CollagenaseH (Sigma Aldrich-2mg/ml), Trypsin (Sigma Aldrich-0,15%) and DNase I (Sigma Aldrich -100 µg/ml) The gonadal fragments were incubated in the enzymatic dissociation medium for 2 hours (21ºC) with gentle shaking (100 rpm).

Subsequently, the dissociated tissue is filtrated using a cell strainer (40 µm), and 10% FBS is added to stop the enzymatic dissociation. Testicular cells were centrifuged at 400G for 5 minutes (21ºC), and the final pellet was resuspended in 1 ml of organoid cell culture medium for cell counting.

Trypan Blue (an exclusion test) is a stain used to stain dead cells blue while live cells remain unstained. The cell suspensions were mixed with Trypan Blue (dilution factor 4), and only live cell concentration was assessed through a Bürker cell counting chamber (Figure 2.3). For that, 40 squares with 0.1mm^3 were counted in triplicate, allowing the total number of cells/ml to be extrapolated.

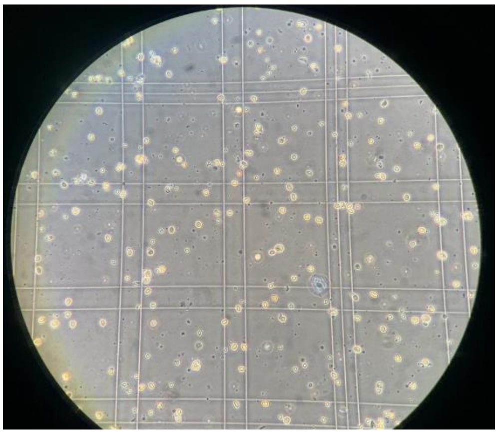


Figure 2.3 Cell Counting and viability through the use of a Bürker cell counting chamber (dead cells stain blue, and live cells do not stain).

2.2 Aggrewell

The Aggrewell plates were composed of 24 wells with 1200 microwells each (Figure 2.4). In this method, the wells are first pre-treated with anti-adherent rinsing solution (Sigma Aldrich-CAT 07010), 500 µl/well, centrifuged at 2000G for 2 minutes, and observed to ensure no bubbles were present. The anti-adherent solution was removed, and the wells were washed with DMEM 500µl/well (Sigma- Aldrich). Subsequentially, the desired number of cells is added to the well and centrifuged at 100 G/10min, allowing cellular aggregation. For optimal results, the density of cells (Table 2.1) was adjusted according to the desired number of cells/microwells and a final volume of 2ml/well was adjusted with the organoid culturing medium. Three aggrewell plates were used during the experiment, with eight wells prepared for each plate. Inside each plate, four wells were prepared utilising 1000 cells/microwell and the remaining wells with 2000 cells/microwell (n = 4). The experiment was kept for seven days, organoids were recorded every two days, and no media was changed (Annex 1.2 Protocol).

Figure 2.4 Aggrewell plate (24 wells with 1200 microwells each).

Table 2.1 Average number of cells per well influence microwell cell density.

Desired number of cells per microwell	Required number of cells/well
100	1.2x10^5
200	2.4x10^5
500	6x10^5
1000	1.2x10^6

2.3. Vitrogel

Vitrogel (TheWell Bioscience) (a synthetic structure that mimics the organic tissue produced by TheWell Bioscience) was used as a scaffold matrix for the testicular organoid cultures together with cell suspension medium (cell pellet + 3x concentrated basic medium) in a ratio of 2:1, displaying a concentration of 1x10^6cells/50µl.

Vitrogel is available in 4 different solutions, vitrogel 1,2,3 and 4. The primary difference between vitrogels is the stiffness (the ability of the vitrogel to be deformed by the pressure applied by the cells), vitrogel 1 has a Pa= Stiffness of 50, and vitrogel 2 and 4 have the same stiffness (between 50 and 300) but differ in bio-functional ligands, allowing vitrogel 2 to be more suitable for gastric cells and vitrogel 4 for cancer cells. Vitrogel 3 has a stiffness of 300, making it the stiffest of the vitrogels.

The first experiment evaluated the performance of the different vitrogels available (which were capable of producing organoids). A 96-well plate accommodated triplicates of each vitrogel, and the same number of cells was utilised for each well (1x10^6 cells/50µl), n is 3. This experiment extended for 21 days, every two days, observations were made to record size, cell movement, cell aggregation, visible spheroids, and number of visible organoids (Annex 1.3 Protocol). The media was changed every seven days.

2.4. Protocol comparison

Through collected data during the experiments, it is necessary to compare the efficiency (time necessary to obtain organoids, size, and average number) of both vitrogel and aggrewell, as well as the internal structure of the organoids (through histology), in order to understand the most optimal method of organoid production.

2.5. Histology of the organoids

To further understand and prove that the organoids produced by sturgeons were indeed fragmented organs, characterising the cells inside the organoids was a mandatory procedure. The cells present in the organoids were studied through histology, allowing the study and identification of germ cells and somatic cells (Sertoli cells and Leydig cells). All histological experiments used organoids kept in aggrewell plates for seven days (with an average size of 100 μm, produced under standard conditions).

The histological procedures used resin has an embedding media and H&E (hematein and eosin) for staining. Due to the sample size, no dehydration is needed during the procedure, the samples are fixed in paraformaldehyde for one hour and embedded in resin for two days. MounThe sample must first be embedded and hardened inside an Eppendorf to mount the resin block samples, which are then collected and embedded again in the block mold and mounted for a future section.

Histological sections are produced with a width of 3µm, and all the sample is used. The histological protocol used during this experiment was produced by Sulliven et al., (2010).

2.6. Transplantation

2.6.1. Preparation of organoids and recipients for transplantation

Prior to transplantation, organoids are retrieved from aggrewell, the concentration is counted by dividing into four a well of a 96-well plate and adding 50 µl of suspended organoids (this procedure will give the number of organoids/50 µl allowing the extrapolation of the total number of organoids). Then, the organoids are labelled with

PKH-26 (Sigma Aldrich) to enable visualisation after the transplant by labelling the lipid membrane of the cells.

To prepare sterile recipients, sturgeon embryos, at one cell stage, are injected with morpholino oligonucleotides against the dead-end gene (*dnd*-MO), which knockdown the expression of a germ-cell specific gene, *dnd*, leading, therefore, to sterility.

The transplantation procedure is performed using a Femtojet® system, and the needles used for micromanipulation are Borosilicate Glass Capillaries (World Precision Instruments, Inc.) 1B100-4.

2.6.2. Organoid Transplantation

All organoid transplantations were conducted into 15 dph sturgeon larvae. The recipient specimens were anaesthetised in 0.03% 2-phenoxyethanol. Cultured organoids were injected into the body cavity of each recipient larva. The needle was inserted at one specific entry point between the bladder and the intestine (Figure 2.5). The visualisation of the organoids entering the body cavity means a successful transplantation. Sturgeon larvae post-transplant were kept in system water and fed with *Artemia*. Both groups were exposed to the same water conditions and the feeding regime.

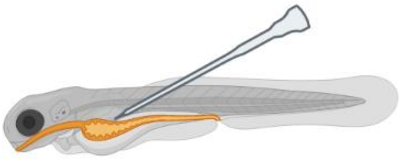


Figure 2.5 Point of injection during organoid transplantation (between the bladder and the intestine).

A total of 50 sturgeon larvae were transplanted in this experiment, and the other 20 were kept as control (sterile larvae). The primary reason for the discrepancy between the control and the variable is the extreme pressure of the micromanipulation (n=20). Because of the large size of the organoids, the injection hole is compromising for the larvae size, leaving them very susceptible to mortality.

In the experiment, ten larvae were harvested at 21 dph, and at the end of the experiment, 45 dph. The larvae were euthanised using 2-phenoxyethanol, dissected and observed for organoid organisation and increase in cell density under a fluorescent magnifying glass, only possible through the detection of PKH-26.

2.7. Statistical analysis

During the experimental period, Rstudios was used to analyse the data collected and the Pvalue was obtained for statistical significance.

2.8. Study design

Throughout 21 days, two major experiments were conducted to study testicular organoids. The first tested four different vitrogels to identify the more suitable for the sturgeon cells, as well as the ones capable of fulfilling the objective of producing organoids. The second experiment tested a different method of organoid production, utilising an aggrewell plate as a scaffold for the organoids. This method tested different cell concentrations (1x10^6cells/500µl and 2x10^6cells/µl) and this method’s ability to create organoids (Figure 2.6). Since no information was available on the species used in this study, individual protocols were created for each experiment (vitrogel and aggrewell). Statistical analysis was made for the respective experiments utilising Rstudios to understand if there were statistical differences between the collected data. In order to gain a better understanding of the organoids' structure, an in-depth analysis was conducted using histology. Additionally, organoid produced by the aggrewell protocol were labelled by PKH-26 (enables the observation of the organoid under fluorescence light) and transplanted into 15dph sturgeon larvae, collection and observation of the larvae was conducted at 21 and 45 dph. Organoid movement and growth were followed during the experiment.

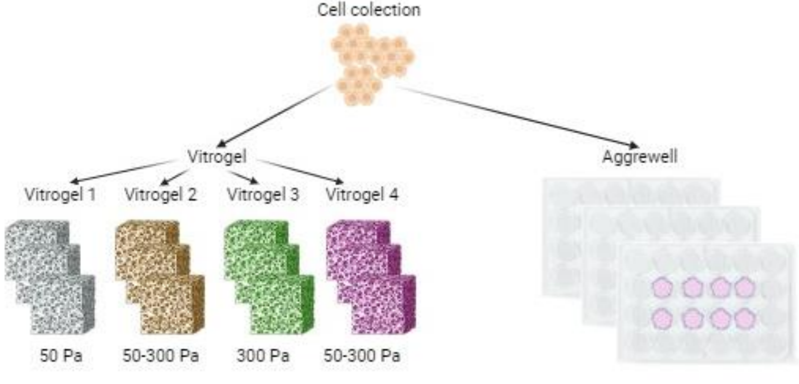


Figure 2.6 Experiment design of vitrogel and aggrewell. Both experiments utilised triplicates, and n=3 for vitrogel and for aggrewell n=8.

3. Results

3.1 Vitrogel Experiment

Vitrogels were compared for 21 days to evaluate the size, cell movement, cell aggregation, visible spheroids, and number of visible organoids. For Vitrogel 1, 20 organoid-like structures (rounded structures with a singular membrane, Figure.3.1 A and B) were observed, showing buds during cell development. For vitrogel 2 and 4, no organoid-like structures were formed, neither round structure nor single membrane were visible, and the cells formed spheroids (grape-like structures) (Figure 3.1C). Vitrogel 3 did not show organoids or spheroids during the 21 days, the cells were not connected (Figure 3.1D). Vitrogel 1 was the only one capable of producing organoid-like structures. This way, vitrogel 1 is the only one used for future analysis, producing, on average, six organoids/well (Table 3.1). The spheroid-like structures grew during the first 15 days of the experiment and then plateaued. The organoid-like structure was only achieved in the remaining days of the experiment (Table 3.2).

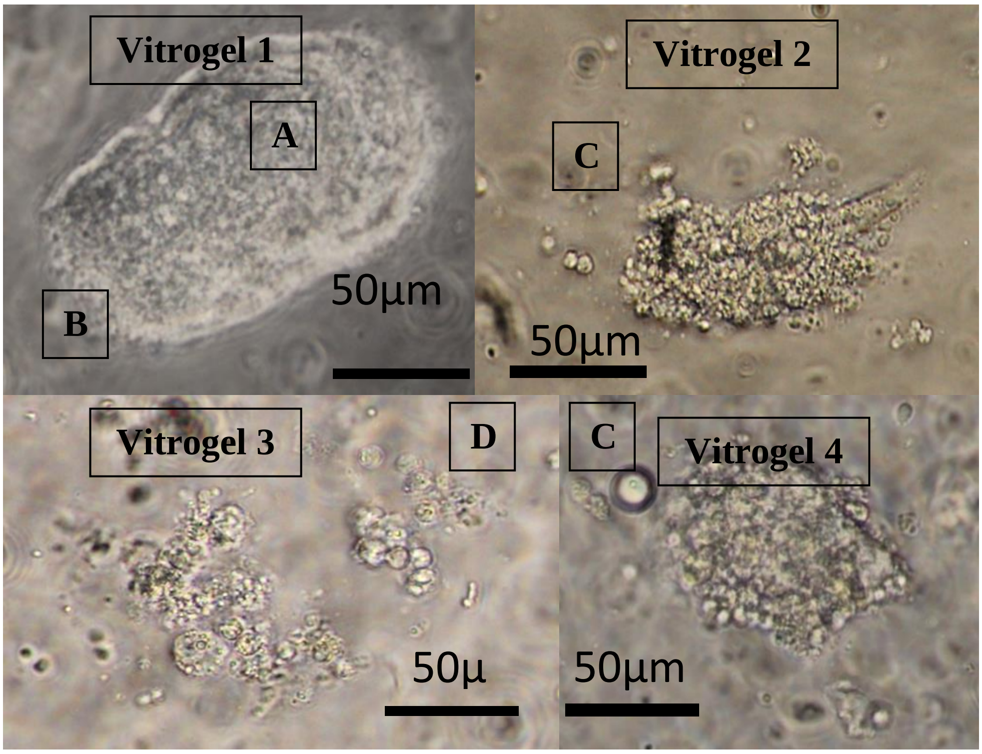


Figure 3.1 Vitrogel 1 (20 days), with visible singular membrane (A), buds (B) and spherical appearance. Vitrogel 2 and 4 (20days), (C) with no membrane or round structure, spheroids were the most organised and Vitrogel 3 (20days), with no organoid or spheroid structures are formed (D), the cells show no organisation and no connection (40x magnification)**.**

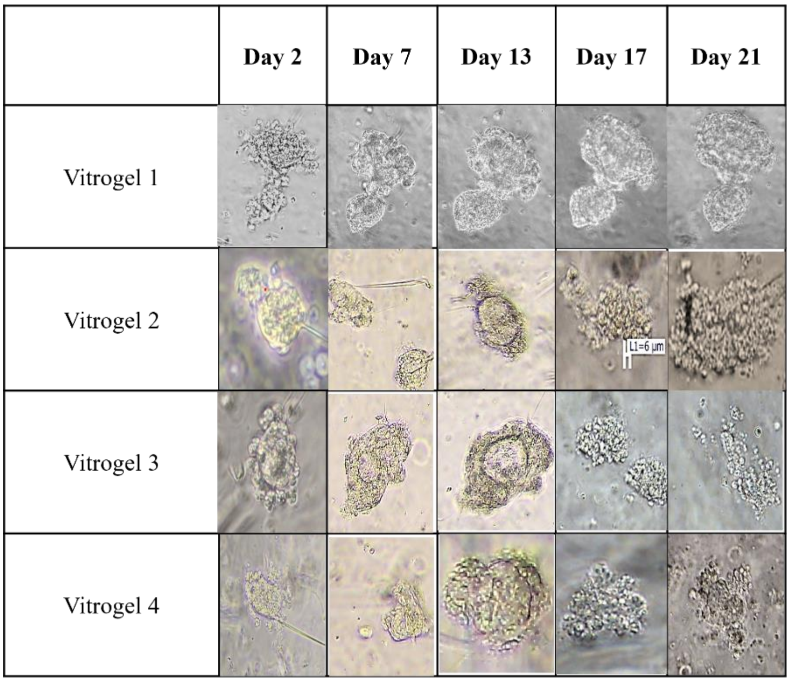
Table 3.1 Observations and development of the vitrogels in the vitrogel experiment.

Day	Cell movement (CM)				Cell aggregation (CA)			
	Vitrogel 1	vitrogel 2	vitrogel 3	vitrogel 4	Vitrogel 1	vitrogel 2	vitrogel 3	vitrogel 4
1	O	O	O	O	O	x	x	x
3	O	O	O	O	O	x	x	x
5	O	O	O	O	O	O	O	O
7	O	O	O	O	O	O	O	O
9	x	O	O	O	O	O	O	O
11	x	O	O	O	O	O	O	O
13	x	x	x	x	O	O	O	O
15	x	x	x	x	O	O	O	O
17	x	x	x	x	x	x	O	x
19	x	x	x	x	x	x	O	x
21	x	x	x	x	x	x	O	x

Day	Spheroid (S)				Organoid (O)			
	Vitrogel 1	vitrogel 2	vitrogel 3	vitrogel 4	Vitrogel 1	vitrogel 2	vitrogel 3	vitrogel 4
1	x	x	x	x	x	x	x	x
3	x	x	x	x	x	x	x	x
5	x	x	x	x	x	x	x	x
7	x	x	x	x	x	x	x	x
9	x	x	x	x	x	x	x	x
11	x	x	x	x	x	x	x	x
13	x	O	x	x	x	x	x	x
15	O	O	x	x	x	x	x	x
17	O	O	x	x	x	x	x	x
19	x	O	x	O	O	x	x	x
21	x	O	x	O	O	x	x	x

O=Presence
x=Abcense

Table 3.2 Organoid development in vitrogel from day 0 to 21.



The organoids visible on the last day of the experiment (day 21) showed an average size of 113.52 µm, and the average number of organoids per well was 6 (Table 3.3). Well, 1 was the one with the lowest average size (Figure 3.2), Pvalue observations showed no statistical significance between the different wells (Pvalues 1 and 2= 0.6046; 1and 3=0.1141; 2 and 3= 0.5415), showing no statistical differences when compared with the remaining wells.

Table 3.3 Number of organoids obtained during vitrogel experiment and size (µm).

Well	Nºorganoids	Size
1	6	118.05
		113.52
		112.53
		99.98
		96.76
		104.18
2	5	92.58
		128.51
		101.34
		167.22
		96.07
3	6	130.37
		167.06
		91.60
		121.18
		132.23
		221.24

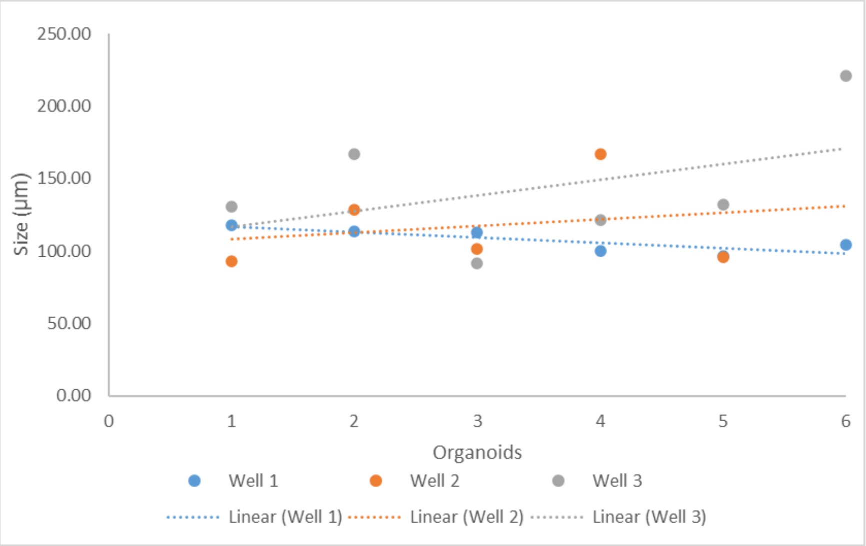


Figure 3.2 Graphic showing organoid size in µm.

3.1.1 Vitrogel Histology

Through the histological procedure, it was possible to identify the structure inside the organoid-like structure (absence or presence of cell organisation) and cell morphology, allowing cell identification. Sertoli cells and spermatogonia were observed during this process, but no Leyding cells were visible (Figure 3.3).

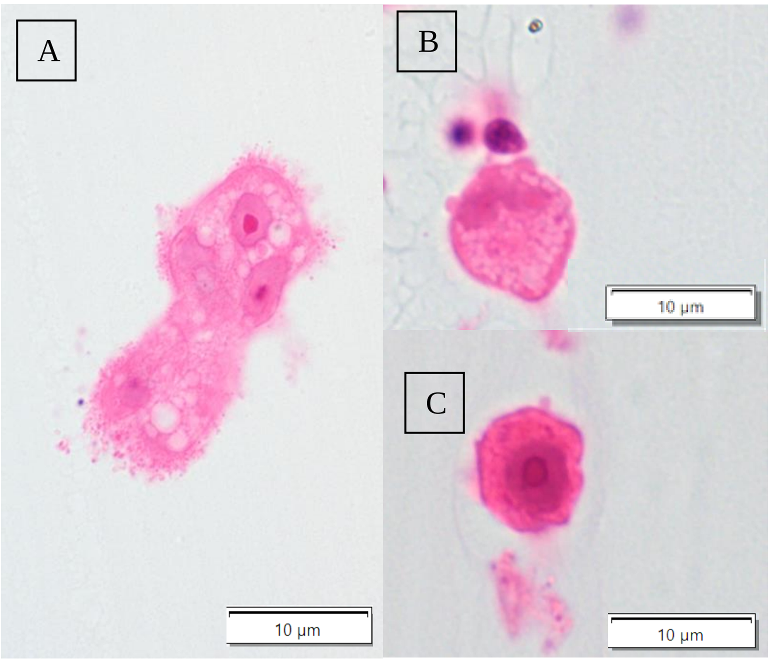


Figure 3.3 Organoids produced in vitrogel 1 collected, concentrated, and fixated in resin 100 x magnification. A-organoid like structure; B-Sertoli cell; C- Undifferentiated spermatogonia.

3.2 Aggrewell Experiment

The aggrewell experiment took place over seven days. The first experiments tested differences in cell concentration (between 1000 and 2000 cells/microwell) to understand the optimal number of cells required to produce organoids. Microwells with 1000 cells show an inability to produce organoid like structures (Figura 3.4 A,B). Meanwhile, the microwells with 2000 cells were successful (Figura 3.4 C,D,E,F,G). Further analysis utilised 2000 cells as standard and produced 1200 organoid-like structures. A total of 30 organoids/plate were randomly selected (n=30), and the length sizes varied between 61.29µm to 311.63µm in length (Figure 3.5). The average size of the organoids was

130.96µm in length (Table 3.4). Statistical analysis (Paired t-test) between the different plates showed no statistical differences between plate A and C, and B-C, plate A and B showed statical differences (A-C Pvalue= 0.1159; B-C, Pvalue=0.126; A-B, Pvalue= 0.005424) indeed. During the recovery procedure, 15% of the organoids are lost.

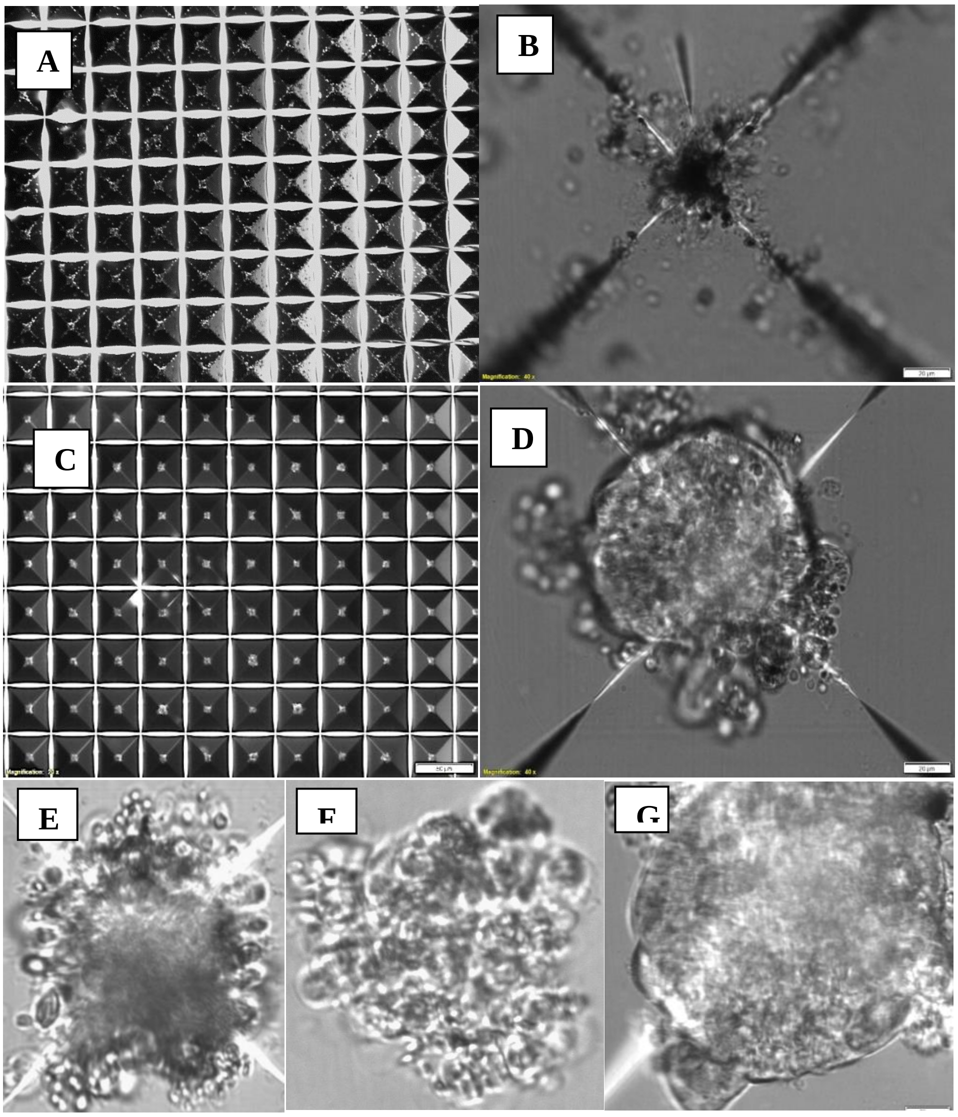


Figure 3.4 A- Observation of the aggrewell plate at 10x magnification; B-Microwell view 40x magnification with no cell aggregation; C- Aggrewell plate using 2000 cells/microwell; D- Microwell view of an organoid like structure at 40x magnification. E- day 1 of culture, no membrane formation and still single cells visible; F- day 3 of aggrewell, cell aggregation visible and a spheroid appearance; G- day 7 of aggrewell culture, a single membrane is visible, and the cell aggregation resembles the structure of an organoid.

Figure 3.5 Graphic showing the different lengths of the measured organoids for the different plates.

Table.3.4 Organoid length at the end of the organoid experiment (7 days) on the different aggrewell plates.

Well	Plate A	Plate B	Plate C
1	142.57	121.28	101.93
2	175.44	72.24	129.77
3	87.72	156.12	101.32
4	87.7	68.37	205.12
5	130.96	61.29	127.79
6	175.14	62.58	135.79
7	170.93	132.91	133.56
8	163.22	93.54	140.61
9	152.87	174.83	159.98
10	125.13	130.96	83.86
11	289.99	107.41	154.8
12	142.56	130.94	96.11
13	90.98	70.32	144.53
14	107.09	120.63	89.66
15	117.39	74.5	173.56
16	203.21	158.05	124.53
17	170.28	205.11	219.96
18	173.52	102.35	208.34
19	182.55	99.34	155.45
20	129.03	102.24	115.56
21	311.63	123.02	149.65
22	108.03	111.49	82.58
23	89.01	92.29	124.49
24	132.9	83.86	104.51
25	148.44	158.04	217.37
26	173.56	167.06	105.37
27	127.09	179.97	157.4
28	156.33	145.15	130.35
29	140.62	169.64	92.88
30	212.35	150.93	128.49

3.2.1 Aggrewell Histology

The histology procedure for the aggrewell organoids showed cell stratification, providing a similar structure to a testicular cist with a single membrane surrounding the cells and a vacant space in the middle of the organoid (Figure 3.6).

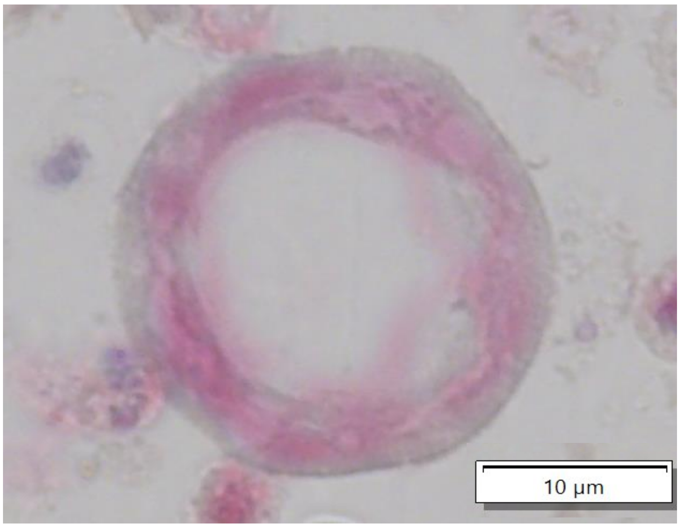


Figure 3.6 Organoid produced in aggrewell, concentrated, and fixated in resin 100 x magnification. Visible cells on the periphery of the organoid, a single membrane, and a cist like appearance.

3.3 Aggrewell Vs Vitrogel

Both experiments showed the capacity to produce testicular organoids. Despite this positive outcome, there were significant differences between both cultures. Aggrewell cultures were shown to be effective, producing 1200 organoids due to the innate structure of the plate.

The nature of the 3D media prevents an accurate visualisation of the total number of organoids inside the vitrogel and an uncontrolled number of organoids formed. The size difference between the organoids in both cultures also had some differences, with the average size for aggrewell being 130.96µm in length and in vitrogel 113µm (with no statistical significance). The period needed to produce the organoids also differs between the cultures, with aggrewell taking 7 days and vitrogel taking 21 days (statistically different). Overall, the aggrewell culture is the most promising for future work.

3.4 Organoid transplantation

As expected, the mortality due to the injection procedure led to a 40% survival rate (Table 3.5), with only 20 individuals following for the next stages of observation of the organoids. During the experiment, the transplanted organoids were visible inside the sterile sturgeons (45 days). On day 21, 9 of the 10 analysed individuals showed positive results (Figure 3.6A), a visible presence of labelled organoids. On day 45 of the remaining 10 individuals, 5 had positive signalling, but in very low amounts. Despite being visible, no organisation of the organoids was ever observed, as well as no increase in cell density (Figure 3.6B).

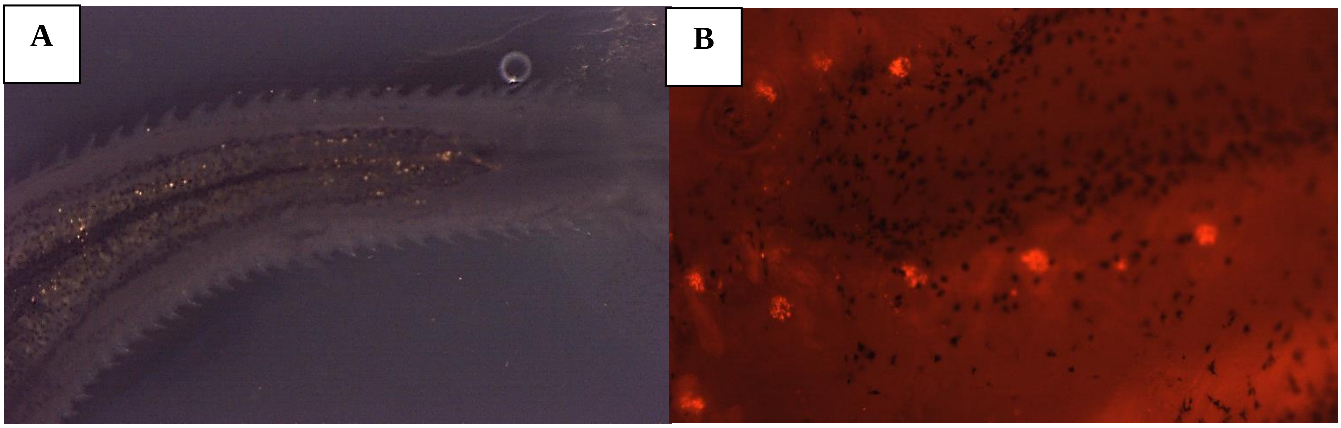


Figure 3.6 Sturgeon juveniles 21 (A) and 45 (B) days after organoid transplantation, showing positive labelled organoids.

Table 3.5 Mortality and survival rate of the larvae post organoid injection**.**

	Day 0	Day 1	Day 2	Total	Total%
Mortality	6	19	5	30	60
Survival	44	25	20	20	40

4. Discussion

Research on cell culture has grown exponentially over the past few decades, proving to be an indispensable tool in understanding biomolecular and biophysical procedures capable of turning single cells into biological tissue (Duval et al., 2017). The use of cell culture to study cell differentiation and epigenetics can push the field of animal reproduction further. The invaluable physical models can unravel how organs are structured mechanically and biologically (Bourdon et al., 2021). Recently, the development of organoids and 3D cultures allowed for an even deeper understanding of cell-to-cell interactions. Wang and co-workers (2020) demonstrated a recent example of how far the research utilising cell culture can go through the culture of murine premeiotic germ cells, and the team was capable of fully observing the cell development from premeiotic cells into fully developed oocytes, with a quality similar to in vivo oocytes.

Mammalian organoids have been the topic of discussion in recent years, and their research allows the recreation of organs and respective diseases in vitro and the exploration of new pharmaceutical drugs and transplantations (Corrò et al., 2020). Researchers must focus on researching fish organoids, specifically testicular organoids, as there is a significant lack of information in these areas compared to mammalian organoids. The only work available at the moment is the in vitro vasculogenic and angiogenesis using zebrafish cells (Ibrahim et al., 2017) and medaka and zebrafish organoids utilising cells for the study of retinal tissue (Zilova et al., 2021). The present thesis project was crucial in clearly defining a fish organoid and laying the foundation for future experiments. Articles have shown that both aggrewell and vitrogel can produce spheroids and organoids, but, unfortunately, there is no consensus on the definition of organoid. Sebrell, et al., (2018) produced human gastric spheroids for their work, utilising vitrogel, which was characterised as having a singular membrane and similar structure and function to the donor gastric organ. Cherne et al., (2021) used the same procedure as in Sebrell work but named the structure organoids (similar characteristic to the structures produced by Sebrell).

The present thesis experiments aimed to end the long-standing debate on the definition of an organoid. The definition of organoid presented during this work states that an organoid is a three-dimensional structure that contains a single membrane and a group of cells capable of differentiation and self-organisation while maintaining the cellular architecture of the original organ. This statement is corroborated by the review paper done by Corrò et al., (2020), where different cell populations have proven capable of producing organoids that follow these specific parameters.

The experiments with vitrogel showed that vitrogel 1, with a stiffness of 50 Pa, was the only one capable of producing testicular organoids in the sturgeon. The most plausible reason is the stiffness of the matrix since it has been proven to be cell-specific, with vitrogel 1, 2, and 3 being more suited to gastric organoids, 1 and 3 used for lungs, 2 and 3 for the brain and 3 and 4 for cancer organoids (TheWell Bioscience). Similar experiments using mammals' organs showed vitrogel as a 3D medium capable of producing organoids with both a membrane and a cell structure like the internal structure of the donor organ (Cherne et al., 2021). The histology for the vitrogel organoids also corroborated the organoid definition, presenting a visible membrane around the cells and structure like the in vivo organ.

The experiment utilising the aggrewell procedure had better results when compared with vitrogel. Aggrewell could produce more organoids in a smaller amount of time. It allowed the production of 1200 organoids after seven days with no statistically significant variations in size with vitrogel.

The aggrewell histology revealed a different aggregation of cells compared with vitrogel. Contrary to Kiani et al., (2021) work in rats, which presented organoids capable of forming organoids with niche-like structures, the experiments with vitrogel showed no visible orientation of the cells or stratification. Aggrewell, on the opposite side, presented a circular structure with a singular membrane with the cells on the periphery and a space in the middle of the organoid. This observation may indicate the formation of a germ cell niche inside the organoid, leading to the hypothesis that cell differentiation is indeed possible and present. This finding corroborates that sturgeon testicular organoid-like structures produced in aggrewell are indeed organoids, unlike the ones produced in vitrogel that do not show a tissue-like structure. The aggrewell organoids follow the initial definition proposed on organoid characterisation, but further studies like immunohistochemistry, in situ hybridisation and qPCR from specific genes need to be explored.

The transplantation of the organoids produced in aggrewell was done as a complementary observation for future organoid work. The sturgeon organoids with an average of 130.96µm were transplanted into sturgeon larvae recipients. The organoids labelled with PKH26 were visible at 21 and 45 dph. Nine out of the ten recipients analysed had positive labelling on day 21, but on day 45, only 5 out of 10 showed any labelling. This may indicate some apoptotic events in these organoids. The transplantation of these organoids showed higher positive results than single-cell transplantations done into sturgeons by Ye et al., (2017). However, larvae suffered higher mortality rates when compared to the ones obtained by the author (23.3%). When comparing organoid transplantation with single-cell transplantation into sturgeon embryos (Saito et al., 2014), the larvae transplanted with organoids had a higher mortality rate and a lower observation rate. The higher mortality rate was expected since the needle needed for organoid transplantation had to be bigger than the one utilized for single cells, allowing a more arduous recovery and a lower survival rate. This preliminary observation opens the possibility that organoids, a collection of cells, are more resistant to post-transplantation stress, but more experiments are needed. Unfortunately, during the 45 days of the experiment, no cell growth was visible inside the host larvae, and no cell migration could be discerned. These results were also different from Saito et al., (2014) and Ye et al., (2017) experiments, where spermatogonia was incorporated in the genital ridge of the recipient. Unfortunately, due to the lower numbers of individuals microinjected during this experiment, there is a need to study organoid transplantation further to understand if it can indeed be a suitable opposition to single-cell transplantation.

5. Conclusion

Vitrogel screening showed that testicular sturgeon cells need to use vitrogel 1 as a 3D media for cell culture to obtain organoids and a minimum of 1x106 cells/50µl. Aggrewell experiments proved that the minimum number of cells capable of producing organoids was 2x106 cells/500µl. Both aggrewell and vitrogel were capable of producing organoid-like structures despite vitrogel inability to produce a similar quantity to aggrewell. Histological observations showed that only aggrewell could obtain a structure similar to a niche testicular structure from both experiments. Organoid transplantation showed that it is possible to transplant organoids and that PKH-26 labelling is visible until 45 dph. Further analysis of organoids is mandatory in order to improve both profitable and non-profitable endeavours. Several endangered animals could benefit from the possible transplantation of organoids into phylogenetically close species and gamete collection from the organoids.

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7. Annex

Protocols

1.1 PROTOCOL - Gonad Dissection and Cell Dissociation Procedure

RECOMMENDED MATERIALS AND REAGENTS

- Sterile scissors, forceps, 15 and 50-ml tubes
- 70 % Ethanol
- Trypsin
- Collagenase H (11074032001)
- FBS (TMS-016)
- DNAse (10104159001)
- PBS /L-15 (L4386)
- Penicillin/Streptomycin, Ampicillin, Amphotericin (P4458; PHR2838; A2942)
- Needles (120mm, 40mm) and 5 ml syringes
- 40 µm Filters

PROTOCOL (using one sturgeon as an example):

- Clean the sterlet (1-2 years) with ethanol and dissect the gonads in sterile conditions under a hood.
- Wash (2x 5 min) the gonads in a solution of PBS, antibiotics and antifungal (10 ml of PBS, 100 µl of Pen/Strep, 50 µl of Ampicillin and 100 µl of Amphotericin.
- Mince the gonads into small pieces with scissors, place them in 15 ml tubes, and add 10 ml of dissociation medium (Table 1).
- Incubate on a shaker with gentle shaking for 2h at RT (room temperature)
- After 2h, dissociate the remaining tissue with a needle and syringe and filter the cells with a 40 µm filter.

- Add 100 µl of FBS to stop the dissociation and centrifuge at 400 RCF for 5 minutes.

- Remove the supernatant and add 1 ml of growing medium.

Table 1 Dissociation medium

Dissociation medium

	Volume	Final Concentration
Trypsin	6 ml	0.15%
Collagenase	500 µl	2 mg/ml
FBS	500 µl	5%
DNAse	20 µl	100 µg/ml
Pen/Strep	100 µl	0.10%
Amp	50 µl	50 µg/ml
Amph	100 µl	2.5ug/ml
L-15	2.73 ml	100%
Total	10 ml	

1.2 PROTOCOL – Aggrewell Culture, Organoid Formation After Seven Days

RECOMMENDED MATERIALS AND REAGENTS

- Aggrewell plate (STEMCELL Technology)

- Sterile hood

- Centrifuge for plates/tubes

- Anti-adherent (STEMCELL Technology)

- Sterile pipettes

- Sterile L-15

- Growing medium 1x concentrated (Table 3.2)

PROTOCOL (using an aggrewell plate as an example):

- Open the aggrewell plate, fill the wells with anti-adherent (500 µl), and centrifuge at **2000 RCF for 10 min.**

- Check under microscope for bobbles inside the wells. If **present, centrifuge again.**

- Collect 490µl of the anti-adherent (is reusable**) without touching** the bottom of the well.

- Add L-15 200 µl to the wells to remove any exec anti-adherent.

- **Check under a microscope for bobbles**

- Introduce a minimum of 250 µl of cell suspension in 1x concentrated growing medium (**cell concentration should be 2.4 million cells/ 250 µl of medium**) inside each well and centrifuge at **400 RCF for 5 min.**

- Check cell density and contamination under microscope and **incubate at 21ºC for seven days**.

- After seven days, the organoids will be fully formed, and further tests can be performed. For continuous observations, replace **50 µl of the media every 7 to 9 days** (organoids can be kept for a period of 21 days with no complications).

Table 2 Growing medium

Sturgeon germ cell culture medium for organoids

	Final Concentration
DMEM	100%
FBS	10%
BSA	2.50%
2-Mercaptoethanol	55 uM
B FGF	10 ng/ml
LIF	25 ng/ml
GDNF	100 ng/ml
Chemically Defined Lipid Conc	0.10%
h ESC	2%
Penicillin Streptomycin	0.10%
Ampicillin	50 ug/ml
Fish serum	1%
NEAA	1%
Acid ascorbic	50 uM
Amphotericin B	2.5ug/ml
Hepes	25mM

1.3 PROTOCOL - Vitrogel 3D Matrix Culture, Organoid Formation After 21 Days

RECOMMENDED MATERIALS AND REAGENTS

- Vitrogel 1, 2, 3 and 4 (different Stiffness). (TheWellBioscience)
- Growth medium 3x concentrated and 1x concentrated (Table 2).
- 96 well-plates
- Sterile pipettes
- Sterile hood
- Incubator

PROTOCOL (using four wells in one 96-well well-plate as an example):

- After cell counting, prepare a solution with 200 µl cell suspension (133.32 µl of vitrogel + 66.64 µl of cell suspension with 3x concentrated medium). The cell concentration should be 2 million cells/50µl of suspension.
- **CRUCIAL STEP: After combining vitrogel and cell suspension, the mixture will solidify.** Before that happens, place 50 µl of the solution in the centre of a well on the plate. The mixture should be added quickly to prevent any loss of biological material. Allow the mixture to harden before proceeding (5 min).
- Check cell density and contamination under the microscope.
- Add 100 µl of 1x concentrated growing medium with a pipet and incubate at 21ºC for 21 days. The medium should be changed every seven days (replace 50 µl of the 1x concentrated medium).
- After 21 days, the organoids should be formed, and further analysis can be performed.