UNIVERSITY OF SÃO PAULO
SCHOOL OF VETERINARY MEDICINE AND ANIMAL SCIENCE

TÚLIO TERUO YOSHINAGA

Development of immunosuppressive methods for gonadal grafting and xenogeneic germ cell transplantation in rainbow trout (*Oncorhynchus mykiss*)

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Original thesis presented to the post-graduation program in Anatomy of Domestic and Wild Animals from the School of Veterinary Medicine and Animal Science in order to obtain the doctoral degree in science.

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FACULDADE DE MEDICINA VETERINÁRIA E ZOOTECNIA

Comissão de Ética no Uso de Animais

CERTIFICADO

Certificamos que a proposta intitulada "Desenvolvimento de Metodologias Imunossupressoras para utilização em enxertos gonadais e transplantes de células germinativas xenogênicas em truta arco-íris (Oncorhynchus mykiss)", protocolada sob o CEUA nº 6973220618 (ID 006413), sob a responsabilidade de **José Roberto Kfoury Júnior** *e equipe; Tulio Teruo Yoshinaga; José Roberto Kfoury Júnior; Ricardo Shohei Hattori; Tulio Teruo Yoshinaga* - que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa científica ou ensino - está de acordo com os preceitos da Lei 11.794 de 8 de outubro de 2008, com o Decreto 6.899 de 15 de julho de 2009, bem como com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi **aprovada** pela Comissão de Ética no Uso de Animais da Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo (CEUA/FMVZ) na reunião de 17/04/2019.

We certify that the proposal "Development of immunosuppressive methods for testis graft and xenogeneic germ cell transplantation in rainbow trout (Oncorhynchus mykiss)", utilizing 360 Fishes (males and females), protocol number CEUA 6973220618 (ID 006413), under the responsibility of **José Roberto Kfoury Júnior** *and team; Tulio Teruo Yoshinaga; José Roberto Kfoury Júnior; Ricardo Shohei Hattori; Tulio Teruo Yoshinaga* - which involves the production, maintenance and/or use of animals belonging to the phylum Chordata, subphylum Vertebrata (except human beings), for scientific research purposes or teaching - is in accordance with Law 11.794 of October 8, 2008, Decree 6899 of July 15, 2009, as well as with the rules issued by the National Council for Control of Animal Experimentation (CONCEA), and was **approved** by the Ethic Committee on Animal Use of the School of Veterinary Medicine and Animal Science (University of São Paulo) (CEUA/FMVZ) in the meeting of 04/17/2019.

Finalidade da Proposta: Pesquisa

Vigência da Proposta: de 08/2018 a 06/2022 Área: Anatomia dos Animais Domésticos E Silvestres

Origem:	Não aplicável biotério						
Espécie:	Peixes	sexo:	Machos e Fêmeas	idade:	1 a 730 dias	N:	330
Linhagem:	Jordanense, shasta, albino.			Peso:	1 a 2000 g		
Origem:	Não aplicável biotério						
Espécie:	Peixes	sexo:	Machos e Fêmeas	idade:	1 a 730 dias	N:	30
Linhagem:	Landlocked			Peso:	1 a 2000 g		

Local do experimento: Unidade de Pesquisa e Desenvolvimento de Campos do Jordão

São Paulo, 17 de abril de 2019

Profa. Dra. Anneliese de Souza Traldi
Presidente da Comissão de Ética no Uso de Animais
Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo

Roseli da Costa Gomes
Secretária
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Evaluation Sheet

Author: YOSHINAGA, Túlio Teruo. **Development of immunosuppressive methods for gonadal grafting and xenogeneic germ cell transplantation in rainbow trout (*Oncorhynchus mykiss*).** 2022. Ph. D. Thesis – School of Veterinary Medicine and Animal Science, University of São Paulo, São Paulo, 2022.

Original thesis presented to the post-graduation program in Anatomy of Domestic and Wild Animals from the School of Veterinary Medicine and Animal Science in order to obtain the doctoral degree in science.

Date: _____/_____/_____

Examination Board

Prof. Dr. _____

Institution: _____ Verdict: _____

Prof. Dr. _____

Institution: _____ Verdict: _____

Prof. Dr. _____

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Dedicatory

I would like to dedicate this work to all the people who were with me in this journey, which without their support I would never reached so far in my life. My parents Antônio and Valéria, my brother Thomas, my sister Tiemi, and my brother Tássio. My supervisors, Dr. José Roberto Kfoury Júnior and Dr. Ricardo Shohei Hattori, and my family from the Salmonid Experimental Station at Campos do Jordão, Drª Yara Aiko Tabata, Drª Neuza Sumico Takahashi, Dr. Ricardo Yasuichi Tsukamoto, Rosana Aparecida da Silva Lopes, Luiz Roberto da Silva, and Antônio Donizete da Silva

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Abstract

YOSHINAGA, T.T.; **Development of immunosuppressive methods for gonadal grafting and xenogeneic germ cell transplantation in rainbow trout (*Oncorhynchus mykiss*)**

In the recent years, germ cell transplantation and the testicular tissue grafts have been used as reproductive biotechnology for commercial and endangered fish species. However, when performed in farther-related species, the donor-derived gametes production is lower or does not occur, while allo- and xenogeneic testis grafts are rejected by the host. The use of immunosuppressive treatment could improve this technology, although their effects on fish immunology and reproduction remain poorly understood. In this study, we aimed to evaluate the use of immunosuppressive treatments in xenogeneic germ cell transplantation and testicular allografts in rainbow trout, as well as the side effects of such substances on male reproduction. Rainbow trout transplanted with Atlantic salmon germ cells were treated with tacrolimus through fed. Our results demonstrate that the administration route used was not able to sustain immunosuppression in the fish larvae, and animals that presented high expression of immune-related makers expression presented lower germ cell counts at the end of the treatment. In addition, tacrolimus treatment did not affect donor-derive spermatozoa production of transplanted animals. Moreover, an immunosuppressive therapy composed by tacrolimus and mycophenolate mofetil mixture demonstrated immunosuppressive effects and reduced acute inflammation in subcutaneous testis allografts in rainbow trout. Nevertheless, histological, and qRT-PCR analysis revealed that allografted testis were severed damaged in treated animals and presented fewer spermatogonia cells in the allografted testis. In addition, this immunosuppressive therapy was also tested in adult rainbow trout to access the effects on male fertility. Rainbow trout males treated during four-weeks demonstrated lower milt volume, but normal sperm concentration compared to control group. Furthermore, immunosuppressive therapy did not affect the fertilization and hatchling of the progeny produced by the treated males. In summary, this study demonstrated that immunosuppression of the fish host may be a suitable approach to improve reproductive biotechnologies, especially in farther-related species. More importantly, the use of immunosuppressive therapy did not affect spermatogenesis in rainbow trout males and probably does not impair transplanted germ cells and gametogenesis of the treated animals.

Keywords – Surrogate broodstock, testis graft, immunosuppression, tacrolimus, mycophenolate mofetil

Resumo

YOSHINAGA, T.T.; **Desenvolvimento de metodologias imunossupressoras para utilização em enxertos gonadais e transplantes de células germinativas xenogênicos em truta arco-íris (*Oncorhynchus mykiss*)**

O transplante de células germinativas e enxertos de testículo em peixes têm sido utilizados como biotecnologia reprodutiva para espécies comerciais e ameaçadas de extinção nos últimos anos. No entanto, quando realizados em espécies distantes filogeneticamente, a produção de gametas derivados do doador é baixa ou não ocorre, enquanto enxertos alogênicos e xenogênicos de testículo são rejeitados pelo hospedeiro. O uso de tratamentos imunossupressivos podem melhorar estas tecnologias, embora os efeitos sobre o sistema imunológico e na reprodução dos peixes permanecem pouco conhecidos. Dentro desse contexto, o objetivo deste estudo foi avaliar o uso de tratamento imunossupressivo em transplante de células germinativas xenogênicos em truta arco-íris e em enxertos subcutâneos de testículo alogênicos, bem como os efeitos deste tratamento sobre a reprodução de machos tratados. Trutas arco-íris transplantadas com células germinativas de salmão do Atlântico foram tratadas com tacrolimus através da ração. Nossos resultados demonstraram que a via de administração usada não foi capaz de sustentar a imunossupressão de alevinos transplantados e os animais que apresentaram alta expressão de marcadores do sistema imunológico apresentaram baixa quantidade de células germinativas ao final do tratamento. Além disso, o tratamento com tacrolimus não afetou a produção de espermatozoides derivados das células de salmão do Atlântico nos animais transplantados. Em outro experimento, uma terapia imunossupressora composta por tacrolimus e micofenolato de mofetila demonstrou efeitos imunossupressores e reduziu a inflamação aguda em enxertos alogênicos subcutâneos de testículo em truta arco-íris. No entanto, análises histológicas e de expressão gênica revelaram que o testículo enxertado estava severamente danificado nos animais tratados e apresentaram poucas células germinativas. Além do mais, esta combinação também foi testada em animais adultos para verificar os efeitos sobre a reprodução dos machos de truta arco-íris. Os animais tratados apresentaram menor volume de sêmen, mas a concentração espermática foi normal comparado com o grupo controle. Mais importante, esta terapia imunossupressora não afetou a fertilização e a eclosão da progênie produzida pelos animais tratados. Em resumo, este estudo demonstrou que a imunossupressão do hospedeiro pode ser opção viável para melhorar as tecnologias reprodutivas, especialmente em espécies mais distantes. Além disso, o tacrolimus e o micofenolato não afetaram a espermatogênese de machos de truta arco-íris e provavelmente não prejudicam as células transplantadas nos hospedeiros tratados.

Palavras-chave – Reprodutores substitutos, enxertos de testículo, imunossupressão, tacrolimus, micofenolato de mofetila

Content

1. General introduction

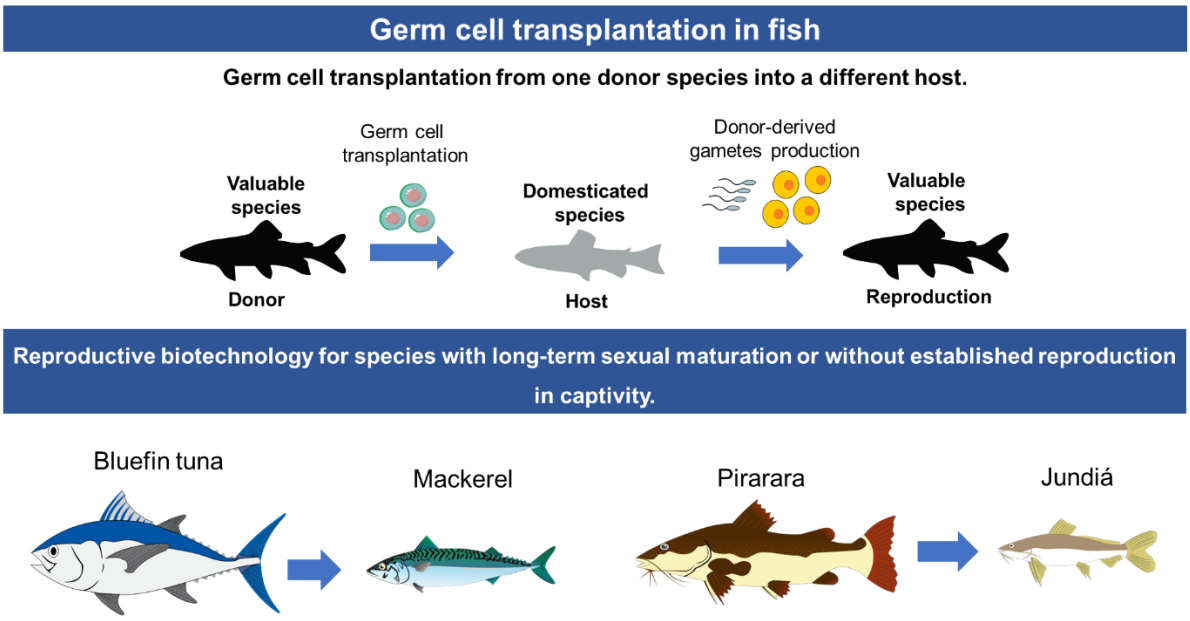
1.1. Germ cell transplantation in fish

Germ cells comprise a cell population responsible to transmit the genetic information from one species to their progeny (CINALLI; RANGAN; LEHMANN, 2008; PELOSI; FORABOSCO; SCHLESSINGER, 2011). The germ stem cells originate during early embryonic stages which will further differentiate in the gametes after the sexual maturation of the organism (HANSEN; PELEGRI, 2021). As well as other stem cells populations, the germ stem cells present two major proprieties, which are self-renewal, and high plasticity (POTTEN, 1992; WAGERS; WEISSMAN, 2004). The first one allows the maintenance of part of the stem cell population at an undifferentiated state, while the other part will follow their normal development into gametes (MARINIELLO *et al.*, 2019). The second one enables the germ cells to survive in a different environment from their original one, such as artificial conditions or transplanted in other organisms (TANAKA, 2016). These characteristics allows germ stem cells to be transplanted into different organisms and to differentiate in functional gametes, as demonstrated in mammals and birds (BRINSTER; ZIMMERMANN, 1994; HONARAMOOZ; MEGEE; DOBRINSKI, 2002; TAJIMA *et al.*, 1993).

In fish, germ cell transplantation was originally performed between rainbow trout (*Oncorhynchus mykiss*) and masu salmon (*Oncorhynchus masou*) (TAKEUCHI; YOSHIZAKI; TAKEUCHI, 2003, 2004). Surrogate broodstock technology, as it was known, consists of transplanting donor germ stem cells into a surrogate host, which will later produce donor-derived gametes (GOTO; SAITO, 2019; YOSHIZAKI; YAZAWA, 2019). Several fish species were reproduced through germ cell transplantation such as pejerrey (*Odontesthes bonariensis*) (MAJHI *et al.*, 2009, 2014), Nile tilapia (*Oreochromis niloticus*) (FARLORA *et al.*, 2014; LACERDA *et al.*, 2010), Yellowtail (*Seriola quinqueradiata*) (MORITA *et al.*, 2012, 2015), medaka (*Oryzias latipes*) (SEKI *et al.*, 2017), zebrafish (SAITO *et al.*, 2010; WONG *et al.*, 2011), fugu (*Takifugu rubipres*) (HAMASAKI *et al.*, 2017), Atlantic salmon (*Salmo salar*) (HATTORI *et al.*, 2019), and more recently common carp (*Ciprinus carpio*) (FRANĚK *et al.*, 2021). Through this technology it is possible to fasten the reproduction of larger species with long sexual maturation period, reducing costs related to the maintenance of breeding stocks (HIGUCHI *et al.*, 2011; MORITA *et al.*, 2012; YOSHIZAKI; YAZAWA, 2019). Furthermore, endangered species with unknown reproduction can also be reproduced by a host species with

known and established reproduction techniques (DE SIQUEIRA-SILVA *et al.*, 2018; MAJHI; KUMAR, 2017).

Figure 1 – Germ cell transplantation scheme and its application in fish



Source: YOSHINAGA, T.T, 2022

The germ cell transplantation can be performed in blastocyst embryos, newly hatched embryos, and adult animals. After the transplant, the donor germ cells will become part of the blastocyst germline, while, in newly hatched embryos, they migrate from the coelomic cavity towards to the genital ridges or from the lumen to the cortical region of the adult gonads (MAJHI *et al.*, 2009; SAITO *et al.*, 2008; TAKEUCHI; YOSHIZAKI; TAKEUCHI, 2003). Then, the transplanted cells will colonize the host gonad, proliferate by mitosis, followed by the meiotic cell division until their fully development into spermatozoa or oocytes, according to the phenotypic sex of the host (OKUTSU *et al.*, 2006b; WONG *et al.*, 2011). Moreover, another method for the surrogate production of gametes is based on grafting the entire testis or testicular fragments directly in adult animals (BHATTA *et al.*, 2012; NAGLER *et al.*, 2001). The grafted testis develops along with the sexual maturation of the animal, being capable to differentiate and produce viable gametes (HAYASHI; SAKUMA; YOSHIZAKI, 2018).

Thus, surrogate broodstock technology comprises a great reproduction biotechnology that can be applied for the preservation of endemic and endangered species (DE SIQUEIRA-SILVA *et al.*, 2018; MAJHI; KUMAR, 2017), to study the reproductive biology of unknow species (OCTAVERA; YOSHIZAKI, 2020; SILVA *et al.*, 2016), and for the reproduction of

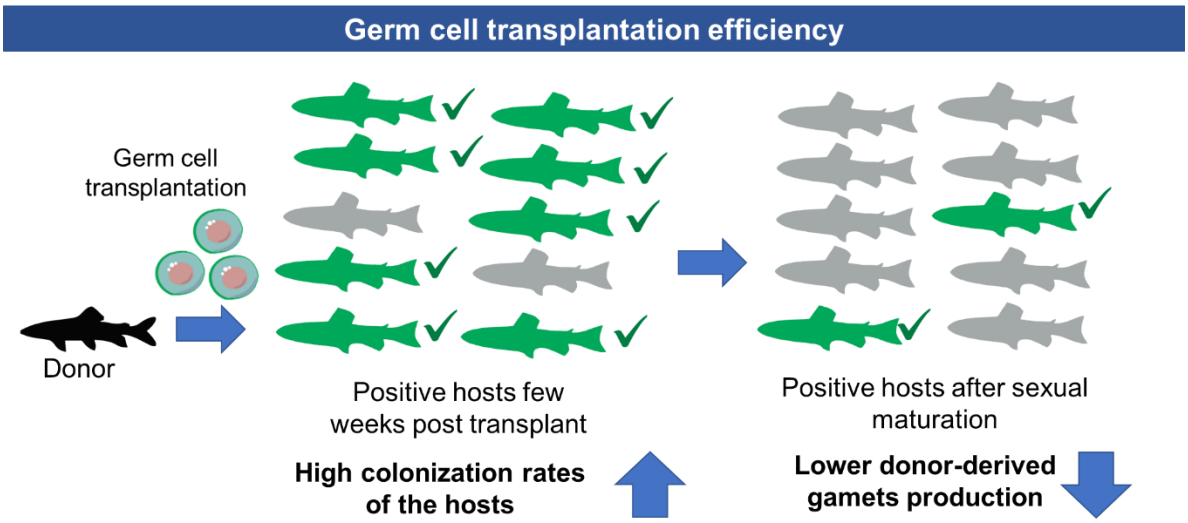
larger species or the ones with long reproductive cycle (KAWAMURA *et al.*, 2020; PŠENIČKA *et al.*, 2015).

1.2 Germ cell transplantation efficiency and fish gametogenesis

Germ cell transplantation was successfully demonstrated in embryos and adult animals (YOSHIZAKI; YAZAWA, 2019). In the beginning, diploid animals were initially used as hosts (OKUTSU *et al.*, 2006a; TAKEUCHI; YOSHIZAKI; TAKEUCHI, 2003, 2004). However, diploid animals are capable to produce their own gametes and hence affect the donor-derived gametes production from the transplanted cells. In order to avoid this problem, sterile or germ cell-less animals are the best hosts for transplantation (GOLPOUR *et al.*, 2016).

Triploid animals are usually sterile due to problems in the meiotic division during the gametogenesis (PIFERRER *et al.*, 2009). In salmonids, triploid female is fully sterile, while the triploid male produces aneuploid spermatozoa, which retains the capacity to fertilize oocytes, but the embryo fails to complete the development and die prior to hatch (LEE *et al.*, 2013). Triploid rainbow trout was already used as host for germ cell transplantation and proved to be a suitable host for different salmonids (HATTORI *et al.*, 2019; OKUTSU *et al.*, 2007). In addition, sterile animals generated by hybridization or by genetic manipulation were also used as host and produces only donor-derive gametes (FRANĚK *et al.*, 2021; KAWAMURA *et al.*, 2020; OCTAVERA; YOSHIZAKI, 2019). The sterility in hybrid animals is also caused by problems in the germ cells meiotic division (XU *et al.*, 2019; YOSHIKAWA *et al.*, 2018), while by the genetic manipulation, the germ cells are prevented to develop during embryonic stages (FUJIHARA *et al.*, 2022; WARGELIUS *et al.*, 2016). Regardless of the host options, several studies reported that transplanted animals did not produce donor-derived gametes, even though the transplanted germ cells being observed in the host gonads after the transplant (HIGUCHI *et al.*, 2011; MARINOVIĆ *et al.*, 2022; PACCHIARINI; SARASQUETE; CABRITA, 2014; SAITO; PSENICKA, 2015; YAZAWA *et al.*, 2010), which suggests that a key factor is missing or limiting the application of germ cell transplantation in different fish species. For instance, physiological and/or immunological compatibility between donor and host may be some of the possible causes.

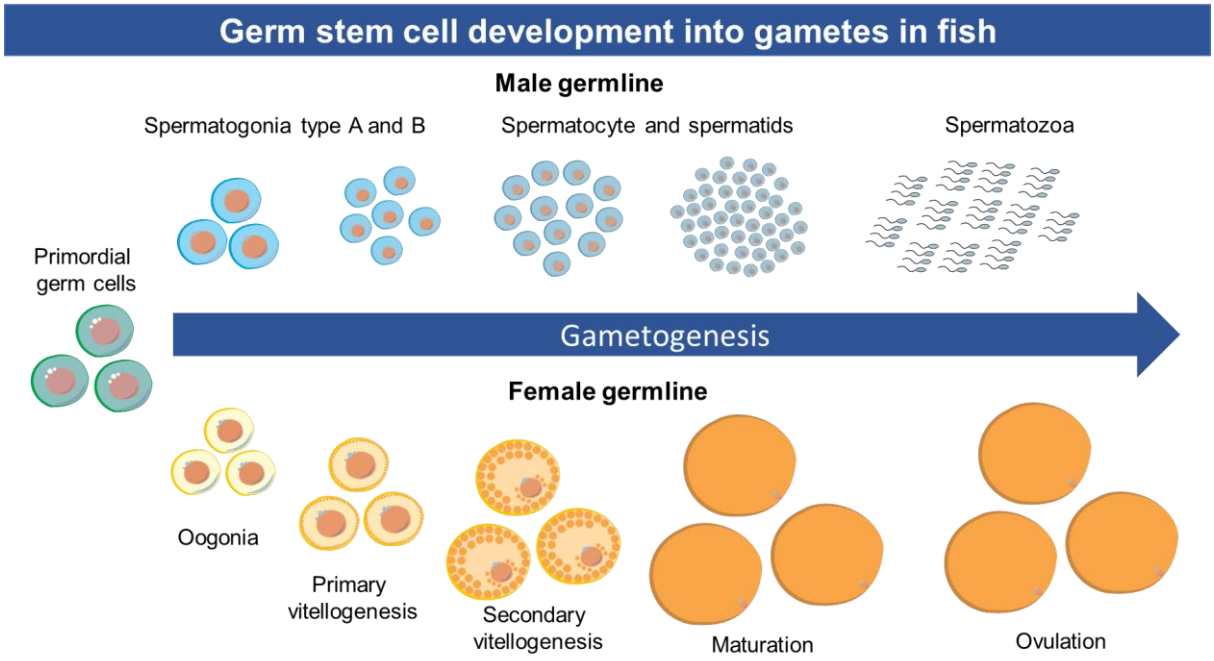
Figure 2 – Germ cell transplantation efficiency scheme



Source: YOSHINAGA, T.T, 2022

The gametogenesis in the transplanted animals may depend on a certain physiological compatibility between donor and host. The granulosa, theca, Sertoli, and Leydig cells are the somatic cells responsible to receive the hormonal stimuli from the hypophysis to start the gametogenesis, also nurture and regulate the gametes differentiation in ovaries and testis, respectively (LUBZENS *et al.*, 2010; SCHULZ *et al.*, 2010). The intrinsic interaction between the donor transplanted cells with these somatic cells may be fundamental to the proper development of transplanted cells in viable gametes, especially in farther-related species. Currently, functional oocytes were only obtained from transplants between different genus, while viable spermatozoa were obtained in inter-order and inter-family transplants (HATTORI *et al.*, 2019; SILVA *et al.*, 2016; ZHOU *et al.*, 2021). In case of oogenesis, the transplanted germ cells receive more maternal elements such as the vitellogenin, the chorionic elements, and high quantities of maternal mRNA, differently from spermatogenesis which consist of a simpler process.

Figure 3 – The development stage of gametogenesis in fish



Source: YOSHINAGA, T.T, 2022

The oogenesis in fish comprises a very complex mechanism and is widely diversified depending on the species (KAGAWA, 2013). The oogenesis starts during embryonic stages with the differentiation of the primordial germ cells into oogonia stem cells, followed by the transition to primary oocytes and the beginning of meiosis (GRIER; URIBE; PATIÑO, 2009). Primary oocytes undergo primary vitellogenesis whereby growth in size, accumulates nutrients, maternal RNA for the embryo development, and completes all the cellular and non-cellular involucres (LUBZENS *et al.*, 2010). During this process, the meiosis I remains stopped at the diplotene stage until the sexual maturation of the female, when in fact the maturation stage begins. This phase is marked by the end of the meiosis I division, originating the secondary oocyte and the first polar corpuscle. From this moment, the oocyte starts its secondary vitellogenesis and the meiosis II, remaining stopped in the metaphasis II until the fertilization (KAGAWA, 2013; READING *et al.*, 2018). After being fertilized by the spermatozoa, the oocyte finishes its meiosis II and release the second polar corpuscle.

Differently from oogenesis, spermatogenesis consists of a simpler process and conserved among vertebrate animals (URIBE; GRIER; MEJÍA-ROA, 2014). After the sexual maturation of the organism, the spermatogenesis includes three very distinct phases: the mitotic proliferation, the meiotic differentiation, and the spermiogeneic phases (SCHULZ *et al.*, 2010). In the first one spermatogonia undergo several mitotic divisions to expand the spermatogonia

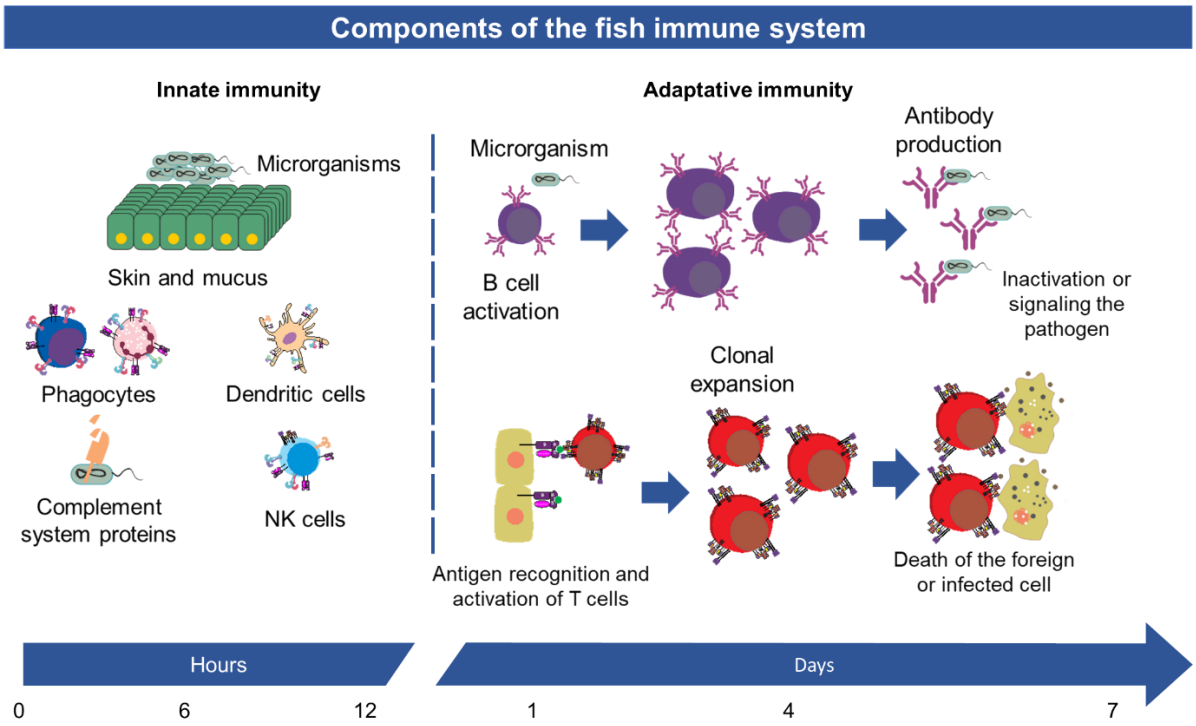
type A and B cell numbers (LACERDA; COSTA; DE FRANÇA, 2014). In the second phase, B spermatogonia cells undergo the first meiotic division, differentiating into primary and secondary spermatocytes. Then, the second meiotic cell division occurs and the transformation of spermatocytes into haploid spermatids (NÓBREGA *et al.*, 2010). In the last phase, haploid spermatids lost most of its cytoplasm, the nucleus become condensed, and a flagellum is formed transforming into spermatozoa (SCHULZ *et al.*, 2010). At this stage, the spermatogenesis is complete and additional capacitation process will occur during its liberation (KOWALSKI; CEJKO, 2019).

Although, physiological compatibility may be crucial for the differentiation of donor germ cells into functional gametes by the transplanted host, another hypothesis for transplanted animals failed to produce donor-derived gametes rely on the immune rejection mechanisms from the host immune system. At some point during colonization or differentiation, the transplanted cells may be attacked and eliminated by the host immune system. Although the belief that during embryo stages, fish immune system is still not well-developed (MANNING; NAKANISHI, 1996; ZAPATA *et al.*, 2006), immune rejection is very well observed in gonads transplantation and testicular grafts (CLOUD, 2003; NAGLER *et al.*, 2001) being viable only in autografts or using isogeneic lineages, while allogeneic or xenogeneic grafts are rejected by the host immune system in few weeks (CLOUD, 2003; HAYASHI; SAKUMA; YOSHIZAKI, 2018; NAGLER *et al.*, 2001; YOSHINAGA *et al.*, 2021). Thus, immunological mismatch between donor germ cells and host may also be overcome in the application of reproductive biotechnologies.

1.3. The fish immune system

The study of the fish immune system has been important particularly from the evolutionary perspective reveling unknow mechanisms from mammalian immunity (SUNYER, 2013) and more recently to improve fish health in aquaculture industry (ASSEFA; ABUNNA, 2018). The immune system comprises a complex set of cellular and humoral mechanisms responsible for the protection of the individual against harmful agents, such as virus, bacteria, malignant cells, and toxins (BILLER-TAKAHASHI; URBINATI, 2014). As in mammals, the fish immune system present is also divided in innate and adaptative immunity, presenting several similar mechanisms (URIBE *et al.*, 2011).

Figure 4 – Components of the fish immune system



Source: YOSHINAGA, T.T, 2022 adapted from Abbas, A. K., Lichtman, A. H., & Pillai, S. (2016). Cellular and molecular immunology (9th ed.). Philadelphia: Saunders Elsevier.

The innate immunity consists of the skin and mucosal epithelial barriers, proteins from the complement system, and different mononuclear cells such as granulocytes, monocytes, macrophages, dendritic cells, and Natural Killer (NK) cells (SECOMBES; WANG, 2012). In general, its mechanisms precede the adaptative immune responses, activating, determining, and cooperating in different reactions, also helping in the maintenance of the system homeostasis (FEARON; LOCKSLEY, 1996; SMITH; RISE; CHRISTIAN, 2019). Furthermore, innate immune responses are capable to recognize pathogen molecular patterns (PAMPs) such as lipopolysaccharide (LPS), peptidoglycans, bacterial DNA, virus RNA, among other molecules present in microorganisms (MAGNADÓTTIR, 2006). After recognizing one of these patterns, innate immune mechanisms are immediately activated and different cells are recruited to kill the infectious agent or to inhibit its activity (LIESCHKE; TREDE, 2009).

On the other hand, the adaptive immunity is mediated by high specialized cells and its mechanism of action includes genes, proteins, biochemical processes that provides the necessary information to respond properly against a particular antigen (URIBE *et al.*, 2011). Moreover, the adaptative immunity develops immunological memory after an immune response and improve its action in case of a new infection (STOSIK; TOKARZ-DEPTUŁA; DEPTUŁA,

2021). Adaptive immune responses are mediated by two main cell populations, B and T cells. B cells can perform phagocytosis and present antigens to T cells, but their main function is the antibody production (SCAPIGLIATI; FAUSTO; PICCHIETTI, 2018). The fish B cells produces three main antibody isotypes, IgM, IgD, and IgT/Z, being systematically present in body fluids, blood, and mucus, playing essential roles for both innate and adaptive immunity in the inactivation of toxins and microorganisms (FILLATREAU *et al.*, 2013).

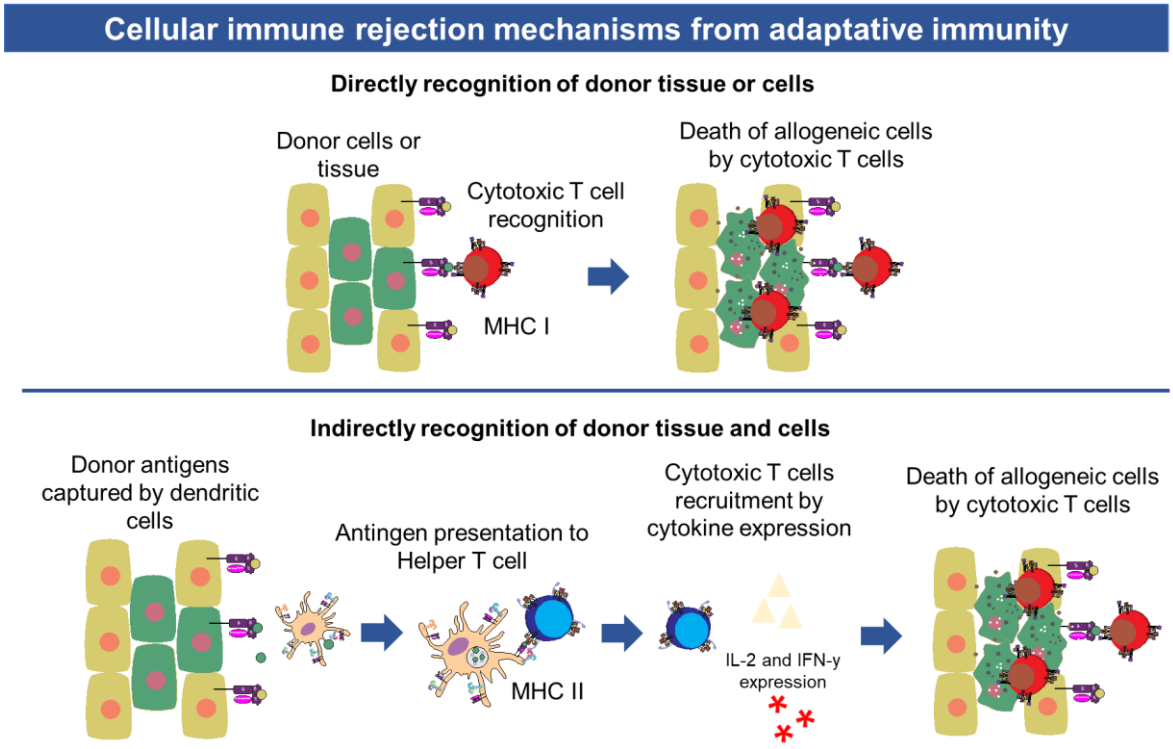
The most important cells from the adaptative immunity are the T cells, and each T cell is equipped with a T cell receptor (TCR) capable to recognize only one antigen presented by a histocompatibility molecule (MHC) (SCAPIGLIATI; FAUSTO; PICCHIETTI, 2018). The MHC molecules are divided in class I and II, being MHC I present in all somatic cells from the organism and recognized by the cytotoxic T cells or $CD8^+$ T cells, while the MHC II are found only in cells that have the antigen presenting function (APC) such as macrophages and dendritic cells, being recognized by the helper T cells or $CD4^+$ T cells (GRIMHOLT, 2016; SHIBASAKI *et al.*, 2015). Through this mechanism, T cells are capable to recognize and destroy virus and bacteria infected cells, malignant or dysregulated cells, and allogeneic or xenogeneic transplanted cells and tissues (NAKANISHI; SHIBASAKI; MATSUURA, 2015).

These mechanisms are always ready to be activated against anything that could do harm or cause damage to the organism and they work together to provide constant protection regardless of the threat, virus, bacteria, or foreign cells. In this case, transplanted germ cells could be rejected by the host immune system as well as any transplanted tissue.

1.4. Immune rejection mechanism from fish immune system

The immune rejection is the mechanism responsible to recognize and destroy allogeneic and xenogeneic donor-derived antigens (INGULLI, 2010). This process is mediated by the interaction of the TCR receptor on the surface of T cells with the donor antigens presented by MHC molecule on foreign cells or by an antigen presenting cell (APC), whereby the TCR recognizes donor antigens attached to the MCH molecule (GRIMHOLT, 2016). Through this mechanism, the T cells are able to distinguish allogeneic and xenogeneic cells from the own organism, in addition to external molecules inside cells when they are infected by some pathogen (CARDWELL; SHEFFER; HEDRICK, 2001; GRIMHOLT *et al.*, 2019).

Figure 5 – Cellular mechanism of immune rejection of the T Cells



Source: YOSHINAGA, T.T, 2022

The first attempt to access cellular immune rejection in fish were performed using skin and scale transplant models (MANNING; NAKANISHI, 1996). These studies described that initially the transplanted tissue lost pigmentation and was gradually covered until form a scar along with a massive invasion of immune cells that progressively destroy the transplanted tissue (HILDEMANN, 1957, 1958). More recently, it was demonstrated that helper T cells are the first ones to infiltrate inside the transplanted tissue, followed by the cytotoxic T cells and B cells, while granulocytes and macrophages appear later to act together with T cells to complete the immune rejection (SHIBASAKI *et al.*, 2015).

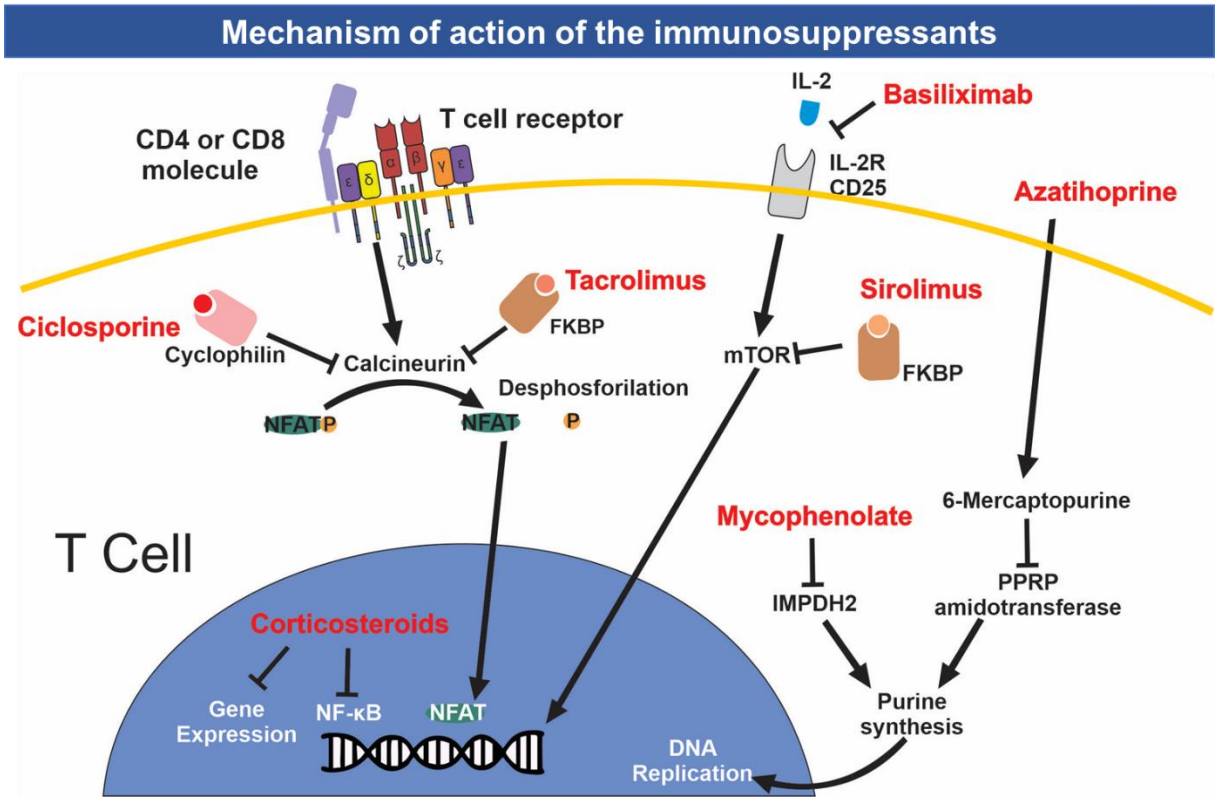
Throughout the immune rejection process, helper T cells acts in the recognition of donor-derived antigens and producing several cytokines such as IL-2 e IFN-γ, which will induce the differentiation and action of cytotoxic T cells and macrophages to destroy the transplanted tissue (MARINO; PASTER; BENICHOU, 2016). The activation and recruitment of cytotoxic T cells and phagocytes is what effectively destroy the transplanted cells and tissues, whereby cytotoxic T cells will inject into the foreign cells several enzymes that will cause its death and decay and phagocytes will help eliminate cellular residuals (NAKANISHI *et al.*, 2011; TODA *et al.*, 2009). Therefore, the immune rejection comprises a complex, specific, and efficient mechanism that involves different cellular populations from the immune system.

In this regard, long-term transplantation studies require the immunosuppression of the individual in order to avoid immune rejection (TAYLOR; ZON, 2009). Although the immune rejection involvement in the lower efficiency of germ cell transplantation remains unknown, the use of immune deficient animals could be used for this purpose (HESS *et al.*, 2013; TANG *et al.*, 2014). Alternatively, the use of immunosuppressive agents can also be used to avoid immune rejection in fish as well as for organ transplantation in humans (HALLECK *et al.*, 2013). Furthermore, recent studies demonstrated immunosuppressive effects on fish immunity also their feasibility and application in transplant studies in fish (XING *et al.*, 2017; YOSHINAGA *et al.*, 2021).

1.5. Mechanism of action of immunosuppressants and their application in transplants

The immunosuppressants are substances that block or inhibit the immune system and have been widely used to avoid immune rejection in organ transplantation (RATHEE *et al.*, 2013). Since their discovery, the immunosuppressants significantly improved the transplantation success and thus the survival rates of host individuals (HALLECK *et al.*, 2013). Moreover, they are also used to treat autoimmune diseases and severe allergies (PENAGOS; DURHAM, 2022; RICHARD-EAGLIN; SMALLHEER, 2018). There are several immunosuppressants with different mechanism of action, such as blocking cell activation, preventing cell proliferation, and inhibiting gene expression (WISEMAN, 2016).

Figure 6 – Immunosuppressive and their mechanisms of action



Source: YOSHINAGA, T.T, 2022

The cyclosporine and tacrolimus (FK506) were originally discovered in fungi, and they have a specific effect on the calcineurin enzyme, a calcium dependent serine/threonine phosphatase (GARCIA *et al.*, 2004). Both immunosuppressants will bind cytoplasmic immunophilins and block the calcineurin activity on the dephosphorylation of the nuclear factor of activated T cells (NFAT) (TEDESCO; HARAGSIM, 2012). The NFAT regulates especially IL-2 cytokine gene expression, essential in the activation of cytotoxic T cells (MACIAN, 2005). Moreover, cyclosporine and tacrolimus were demonstrated to have similar effects on fish immune system. They inhibit leucocyte proliferation *in vitro*, inhibit *il2* gene expression, and reduce leucocytes counts in peripheral blood (CAO *et al.*, 2021; XING *et al.*, 2017). Furthermore, the administration of emulsion-containing tacrolimus was demonstrated to delay immune rejection of spermatogonial cells in subcutaneous testis graft in rainbow trout (YOSHINAGA *et al.*, 2021).

Differently from cyclosporine and tacrolimus, there are immunosuppressants that prevent the cell proliferation such as azathioprine and mycophenolate mofetil (SCHOOT *et al.*, 2020). The azathioprine is a purine analogue molecule (6-mercaptopurine) that inhibits the nucleotide synthesis during the DNA replication (MALTZMAN; KORETZKY, 2003), while

the mycophenolate mofetil inhibits the enzyme inosine-monophosphate dehydrogenase 2 (IMPDH2) present in *de novo* pathway of nucleotide synthesis (ALLISON, 2005). During the mitosis, T cells are extremely dependent on *de novo* synthesis pathway, which is blocked by the mycophenolate mofetil effect preventing T cell proliferation (IPATA, 2011; YIN *et al.*, 2018). There are fewer studies that demonstrate effects of these immunosuppressants on fish immunology. Azathioprine reduces leucocytes counts in peripheral blood and increase apoptosis in spleen and pronephros of Nile tilapia (GOGAL *et al.*, 1999), while the mycophenolate mofetil effects on fish immunity remains unknown.

Although the immunosuppressants have revolutionized the organ transplantation technique in humans, the effects of the majority of these drugs on immune system of fish still very limited, including the respective side effects. Both cyclosporine and tacrolimus cause arterial hypertension, decrease blood flow in the kidneys, and diabetes (BORDA *et al.*, 2014; KLEIN *et al.*, 2002). Other severe effects include acute and chronic nephrotoxicity with significant reduction of glomerular filtration, especially in cyclosporine-treated patients (WHITING *et al.*, 1982). In addition, cyclosporine stimulates renin-angiotensin system causing fibrosis inside the nephrons followed by the kidney failure (LEE, 1997). On the other hand, the use of tacrolimus causes nephrotoxicity, but with a minor scale compared to cyclosporine, as patients that replace cyclosporine by tacrolimus are reportedly to show better kidney functions (PRATSCHKE *et al.*, 1997). Thus, tacrolimus has been considered a better immunosuppressant compared to cyclosporine due to milder side effects and more potent actions (GARCIA *et al.*, 2004). In fishes, some side effects were reported in zebrafish, whereby tacrolimus can cause abnormalities on fin regeneration, while the cyclosporine interfere on the NOTCH signaling pathway disrupting blood vessels formation in embryos (KUJAWSKI *et al.*, 2014; PANDEY *et al.*, 2015). Nevertheless, these effects are not directly associated with the immunosuppression, but the calcineurin inhibition activity by the immunosuppressants.

Regarding to the azathioprine, there are evidences that increases the risk of cancer in transplanted patients (JIYAD *et al.*, 2016), among other side effects such as hair loss, liver dysfunction, and colitis (HIBI *et al.*, 2003). The mycophenolate mofetil cause side effects on the gastrointestinal trait, causing diarrhea and nausea, and also impact the hematological system causing leucopenia and anemia, effects on the urinary trat have also been reported (ZWERNER; FIORENTINO, 2007). These effects are related to the mechanism of action of these immunosuppressants, wherein tissues with high proliferative rates such as the intestinal mucosa, bone marrow, and skin are the most affected (HOUSSIAU *et al.*, 2010). In general,

mycophenolate mofetil have been demonstrated to be safer compared to azathioprine, due to its specific effect on lymphocytes (DOOLEY *et al.*, 2011).

Although side effects are the main concern about the use of immunosuppressive agents as the animal become susceptible to diseases during the treatment, some studies demonstrate their potential and application in reproductive biotechnologies. By identifying the efficacy of these substances, including the side effects, it may be possible to improve and to expand the application of surrogate broodstock technology in fish, as in organ transplantation between humans and also from other mammals to humans.

2. Justificative

The reproduction of commercial and endangered species comprises the main application of the surrogate broodstock technology, due to the expansion and diversification of the aquaculture industry worldwide and the increasing number of the endangered species, respectively. Nevertheless, the low efficiency in the production of donor-derived gametes, probably related to the action of the host immune system, represents a great limitation to establish effectively the surrogate broodstock technology especially in farther-related species. Moreover, the reproductive immunology consists of an unexplored field in fish particularly the immune privilege of the gonad and the interaction between the immune and the reproductive system. In this context, the elucidation of the crosstalk between the immune system and gonads might be fundamental to optimize the protocols of germ cells/gonads transplantation and thus improve the surrogate production of gametes, increasing the understanding of the autoimmune mechanisms related to the spermatogenesis, and the immune system impairment during the reproductive season.

The rainbow trout and the Atlantic salmon are animal models used all over the world and have several methodological tools and database available, such as sequenced genome, wide variety of monoclonal antibodies, and established microinjection and micromanipulation techniques. Furthermore, as we tested before, those species tolerate surgical procedures of testis graft with immunosuppressive treatment. Thus, these two salmonids may be considered suitable experimental models for the execution of this project.

3. General objective

The aim of this study was to evaluate the feasibility of immunosuppressive drugs for suppressing immune rejection mechanisms, with the ultimate goal to improve the efficiency of germ cell transplantation and subcutaneous testis graft techniques in rainbow trout.

3.1. Specific objective

- To evaluate tacrolimus immunosuppression by oral treatment in sterile rainbow trout transplanted with Atlantic salmon germ cells.
- To combine the tacrolimus with mycophenolate mofetil in emulsion solutions and apply in subcutaneous testis allograft in rainbow trout.
- To evaluate the impaction of tacrolimus and mycophenolate immunosuppressive therapy on male reproduction of rainbow trout.

Chapter 1

Tacrolimus immunosuppressive treatment in xenogeneic germ cell transplantation from Atlantic salmon (*Salmo salar*) in triploid rainbow trout (*Oncorhynchus mykiss*)

Tacrolimus immunosuppressive treatment in xenogeneic germ cell transplantation from Atlantic salmon (*Salmo salar*) in triploid rainbow trout (*Oncorhynchus mykiss*)

4.1. Introduction

Surrogate broodstock technology in fish has been performed in several fish species in recent years (JIN *et al.*, 2021). Through this technology is possible to obtain functional gametes from one species using a surrogate host (TAKEUCHI; YOSHIZAKI; TAKEUCHI, 2003, 2004) The technique has been applied for the reproduction of commercial species in order to reduce costs related to the maintenance of breeding stocks and for the reproduction of endangered species using a host with established reproduction in captivity (DE SIQUEIRA-SILVA *et al.*, 2018; MAJHI; KUMAR, 2017; YOSHIZAKI; YAZAWA, 2019).

Surrogate broodstock was initially described in salmonids (TAKEUCHI; YOSHIZAKI; TAKEUCHI, 2003). Since then, the technique has been improved using germ cell-less recipients and combined with other approaches such as cryopreservation and *in vitro* culture of germ cells (IWASAKI-TAKAHASHI *et al.*, 2020; LEE *et al.*, 2013). Nevertheless, there are few unsuccessful trials in some species which the transplanted cells were visualized into embryos right after the transplant, but the animals did not produce donor-derived gametes (DE SIQUEIRA-SILVA *et al.*, 2021; HIGUCHI *et al.*, 2011; PACCHIARINI; SARASQUETE; CABRITA, 2014; SAITO; PSENICKA, 2015; YAZAWA *et al.*, 2010). These results could be related to immune rejection mechanisms from the host organism against the transplanted germ cells.

Teleost fishes have an immune system comparable to mammals and capable to develop complex immunological responses such as immune rejection (SUNYER, 2013). This is a characteristic problem in transplant studies whereby donor cells and tissues are recognized as foreign and destroyed by the recipient immune system (NAKANISHI; SHIBASAKI; MATSUURA, 2015). Although the belief that during embryo stages fish immune system is still not well-developed (MANNING; NAKANISHI, 1996; ZAPATA *et al.*, 2006), fish embryos are capable to develop complexes immune responses against pathogens during egg and embryos stages (CASTRO *et al.*, 2015; CHETTRI *et al.*, 2012). Therefore, the transplanted germ cells could be rejected during the migration, the colonization of the genital ridges, or until the gonad are completely formed.

In order to avoid immune rejection, the use of immunosuppressive agents could be a suitable approach and significantly increase the success of organ transplantation in humans after

their discovery (RATHEE *et al.*, 2013; VAN SANDWIJK; BEMELMAN; TEN BERGE, 2013). These agents inhibit the activation of the immune system, preventing cytokine expression or the cell proliferation (HALLECK *et al.*, 2013). However, their effects on fish immune system are not well understood yet, as well as suitable methods of administration. Some studies demonstrated effects on *in vitro* leucocytes proliferation, reducing leucocyte counts from peripheral blood, and inhibit cytokine expression (PARK *et al.*, 2002; XING *et al.*, 2017; YOSHINAGA *et al.*, 2021). More recently, tacrolimus immunosuppression delayed the immune rejection in testis subcutaneous allografts in adult rainbow trout (*Oncorhynchus mykiss*), which demonstrate their application in reproductive biotechnologies (YOSHINAGA *et al.*, 2021).

Therefore, we hypothesized that immunosuppressive treatment could also have a protective effect on the transplanted germ cells in embryos. Thus, in this study we aimed to evaluate the tacrolimus immunosuppressive effect in rainbow trout (*Oncorhynchus mykiss*) transplanted with Atlantic salmon (*Salmo salar*) germ cells. We also investigated a suitable dosage and whether immunosuppression could be administrated through fed to prevent immune rejection in germ cell transplantation studies.

4.2. Materials and methods

4.2.1. Fish rearing conditions and ethical statements

This study was conducted at the Salmonid Experimental Station at Campos do Jordão, UPD-CJ (Campos do Jordão, Brazil). All animals used were kept in $2m^3$ round tanks with constant water flow at 13 °C (+/- 2 °C) under natural photoperiod conditions. Animals were fed commercial food twice a day during the entire experiment. All procedures were carried out in accordance with the guidelines for the care and use of laboratory animals of School of Veterinary Medicine and Animal Science of University of São Paulo, certificate CEUA-FMVZ-USP # 6973220618.

4.2.2. Atlantic salmon germ cell transplantation in sterile rainbow trout

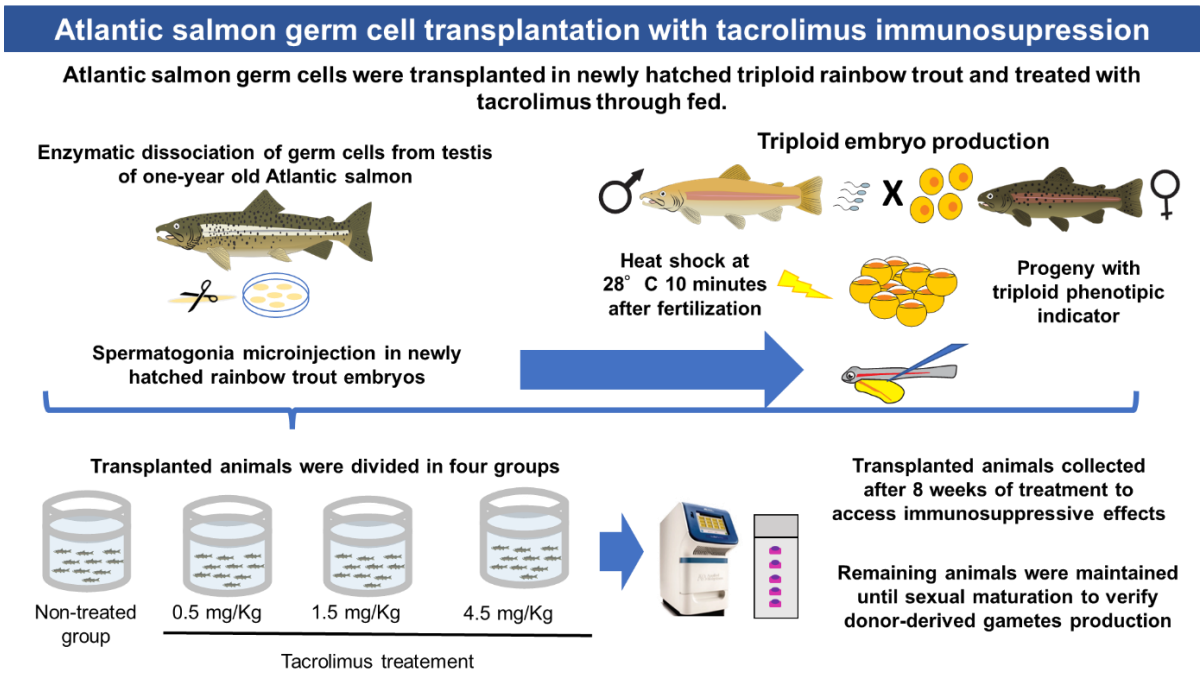
Germ cell transplantation was carried out as described in a previous study (HATTORI *et al.*, 2019). Spermatogonia stem cells were isolated from testis of 8 parr stage males with about one-year age. The testes were dissected and submitted to enzymatic cell dissociation treatment with a PBS buffer (pH 8,2) containing 0,125% of trypsin (Worthington, US), 5% of

Fetal Bovine Serum (Gibco, Invitrogen), 1mM of $CaCl_2$, and 0.01% of DNase (Roche Diagnostics, Germany). After incubation for two hours at 20°C, the resultant cell suspension was filtered through a 45um nylon mesh strainer (Corning Falcon, Germany). The cell suspension was then washed in MEM-5 (Sigma-Aldrich) supplemented with 2.2g/l of $NaHCO_3$ and 5.958g/l of HEPES. Donor cells were labeled with CFSE (Invitrogen), counted in hemocytometer (Improved Double Neubauer Ruling Resistance, Germany), and adjusted to 5.10^5 cells/ml.

Sterile triploid rainbow trout were produced by artificial fertilization of a pooled oocytes from 10 females of Shasta (SS) lineage with milt from dominant yellow albino males. Fertilized eggs were incubated at 10°C for 10 min followed by a heat shock treatment in warm water at 28°C for 20 min. Then, fertilized eggs were transferred to incubators with constant water flow at 10°C until hatching. Triploid individuals were confirmed by the intermediate palomino skin color pattern (HATTORI *et al.*, 2020) (**Figure 7**).

Newly hatched triploid rainbow trout were submitted to anesthesia with benzocaine solution (0.5mg/l) for a few minutes and placed into a petri dish with a 3% agarose gel. Microinjection was performed with a glass needle with 50um diameter coupled to a micromanipulator M152 (Narishige, Japan). Approximately 5000 CFSE-labeled spermatogonia were microinjected in the coelomic cavity of each embryo (n=700). After the transplantation, the embryos were returned to the incubator until the yolk sack absorption.

Figure 7 – Scheme of the germ cell transplantation with tacrolimus immunosuppression



Source: YOSHINAGA, T.T, 2022

4.2.3 Immunosuppressive treatment with orally administrated tacrolimus

After the yolk sack absorption, the transplanted fish started to feed and the treatment with tacrolimus was initiated. Fish were divided in 4 groups, whereby three groups received tacrolimus in four different dosages: 0 (non-treated) 0.5, 1.5, and 4.5 mg/Kg, (**Figure 7**). Commercial fed for rainbow trout were powdered in a blender and mixed with an absolute ethanol containing dissolved tacrolimus. The mixture was put into oven at 37°C until total ethanol evaporation. Fish were the fed during 8 consecutive weeks. Transplanted animals were collected at the end of the treatment to access the immunosuppression and the presence of germ cell in the gonads.

4.2.4 Donor-derived gametes production

Transplanted animals were maintained until sexual maturity. After two years the animals were checked every week and those producing gametes were selected. The gametes were collected for genomic DNA PCR analysis. After the spawning season, the remaining fish were sacrificed and have the gonad collected for histological analysis.

4.2.5. Histological analysis and cell counting

Transplanted embryos eight weeks post-transplant and gonads from transplanted animals after sexual maturation were fixed in Bouin's solution at 4°C for 24 hours and kept in ethanol 70% until processing. Tissues were embedded in paraffin and sliced in 5-µm thickness sections. In order to cover the entire gonad, a total of 9 slides (3 adjacent section/slide) were prepared for each sample, with a distance of approximately 100µm among each other. Transplanted embryos sections were then stained with hematoxylin and eosin (HE) for morphological observation of the gonadal tissue. Germ cells were counted as follow: Images from each section were captured with a CCD Camera (DP73) and analyzed by CellSens software (ver. 1.12; Olympus, Japan), germ cells were counted using the ImageJ Software 1.8v (NIH, USA) with the plugin for cell count.

4.2.6. RNA isolation, qRT-PCR analysis, and genomic DNA PCR analysis

Embryo samples were fixed in RNA later (Invitrogen, USA) during 12 hours at room temperature and stored at -80°C until further analysis. Tissues were homogenized using L-beader 6 cell disruptor tissue homogenizer (Loccus Biotechnology, Brazil), and total RNA was extracted using TRIzol Reagent (Invitrogen, USA). RNA samples concentration was analyzed and measured using the NanoDrop™ 2000 Spectrophotometer. A total of 1 µg of RNA was treated with DNase I amplification grade (Invitrogen, USA), and first strand cDNA was synthesized by SuperScript III (Invitrogen, USA) with $OligoDT_{12\text{-}18}$ primer (Invitrogen, USA). All procedures were performed following manufacturer's instructions.

The expression analyses by qRT-PCR were performed using SYBR Green Master Mix (Applied Biosystems) in the StepOnePlus™ Real-time PCR system (Applied Biosystems) in a 10 µl reaction volume. The primer sequences, amplicon size, and GenBank access numbers are listed in **Table 1**. The two-step qPCR condition was as follow: 40 cycles of 95 °C for 60 s, and 60 °C using the StepOne Software 2.3 version. The transcript abundance was quantified by the Standard Curve Method with four dilution points and normalized using the respective *β-actin* values.

Genomic DNA was extracted by standard saline buffer protocol and subjected to PCR analysis with species-specific primers designed in the *amh* (anti-Müllerian hormone) gene: SsaAmhFw (5'-GCA GTC AAG TTA AGA TAT TGT G-3') and SsaAmhRv (5'-GAA TCT CTA TGG CTT CAA CC-3') for Atlantic salmon and β-actin was used as template control with a set of primers: ActbFw (5'CAA GGC TAA CCG TGA AAA-3') and ActbRv (5'-GCT ACT

CCT TCG TCA CAA CTG CCG-3'). Genomic DNA PCR reaction was performed with EmeraldAmp® GT PCR Master Mix (Takara Bio Inc., Shiga, Japan) using 10-50 ng of genomic DNA. The amplification conditions were as follow: 35 cycles of 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 90 s for Atlantic salmon amh; 35 cycles of 95 °C for 30 s, 66 °C for 30 s, and 72 °C for 90 s for β-actin gene.

Table 1 – Primers used for RT-PCR analysis

Gene name	Primer sequence (5' → 3')	Amplicon size in bp	Genbank accession number
il2	Fw: CATGTCCAGATTCAGTCTTCTATACACC Rv: GAAGTGTCCGTTGTGCTGTTCTC	211	NM_001164065.2
ifnγ	Fw: CCGTACACCGATTGAGGACT Rv: GCGGCATTACTCCATCCTAA	256	NM_001124620.1
cd8b	Fw: GGTTGTACTCCTGTATGCTCCAGA Rv: AGAAGAACTCCTGGCCTCCACAAC	64	NM_001124008.1
cd4-1	Fw: CGAGAGACGATAGATCCAGAGTGGAAAG' Rv: TGTGACTGAGGTACTTGTTTGTGGCAT GA	150	NM_001124539.1
actinb	Fw: GCACTGGTTGTTGACAACGGA Rv: AACCATCACTCCCTGATGCCT	117	NM_001124235.1

4.2.7 Statistical analysis

All data were analyzed in GraphPad Prism 9 (GraphPad Software, USA) using One-way ANOVA, followed by Tukey's multiple comparison test. The level of significance was considered for $p \leq 0.05$. All data were presented as mean ±S.E.M.

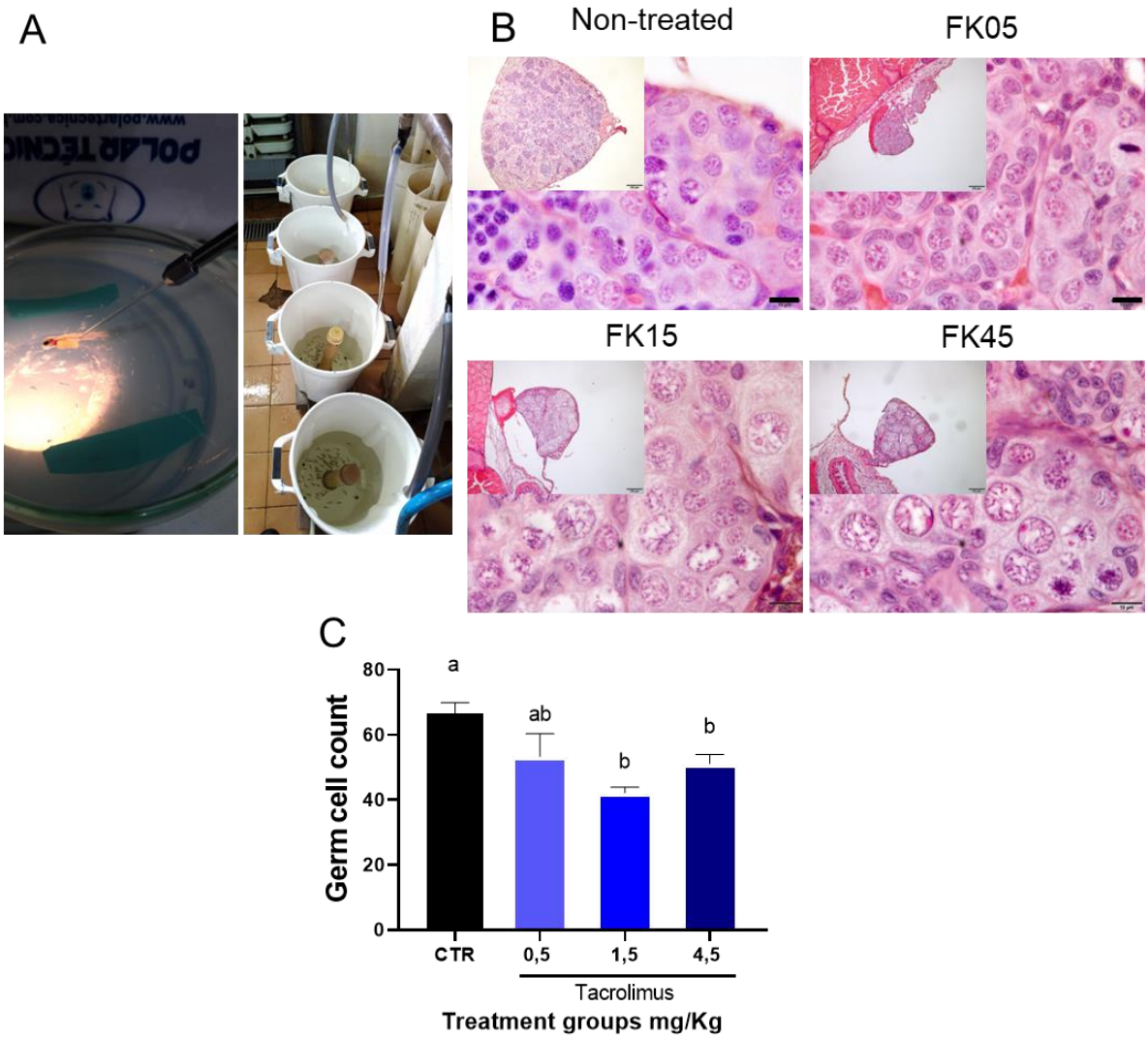
4.3. Results

4.3.1. Germ cell colonization in embryos

Colonization rates of transplanted animals were around 75% in the fish larvae eight weeks after the transplantation (Figure 8A). Histological analysis from transplanted animals showed germ cell presence eight-weeks post-transplant in all four groups (**Figure 8B**). Germ

cells were counted and revealed that non-treated group presented a higher germ cell counts compared to 1.5 and 4.5 mg/Kg tacrolimus treatment.

Figure 8 – Germ cell transplantation and tacrolimus treatment groups.



Source: YOSHINAGA, T.T, 2022

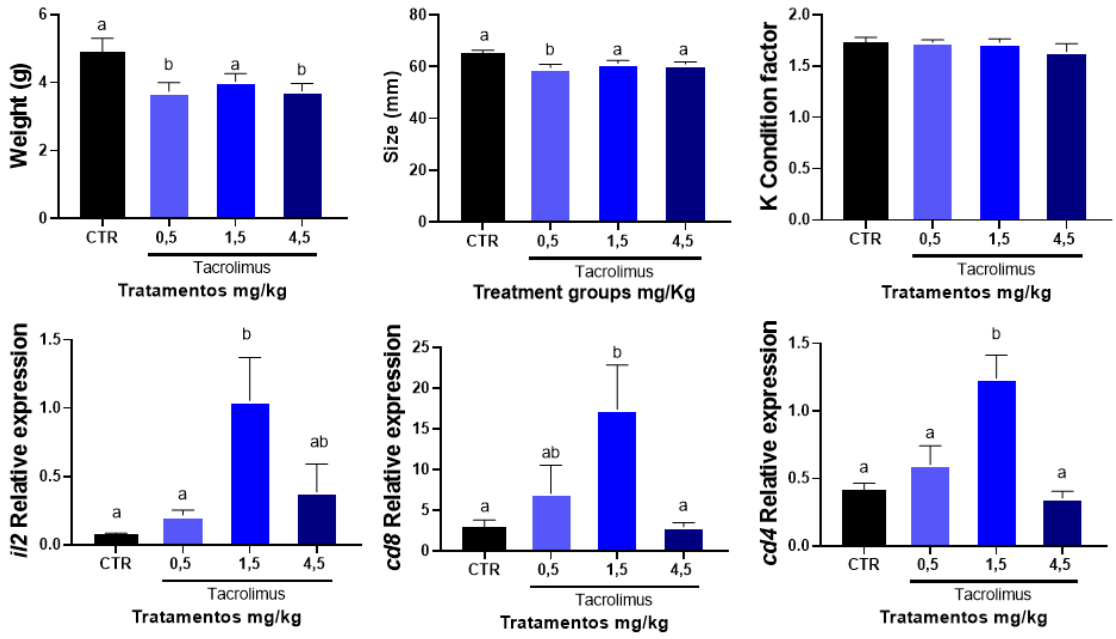
Legend (A) Germ cell transplantation and gonadal histology in tacrolimus treated groups. (B) Histological analysis of the gonads from the transplanted embryos treated with tacrolimus evidencing the gonad and the germ cells niche. Scale bar indicates 10 μm. (C) Germ cell count in transplanted larvae. Different letters represent significantly difference for $p \leq 0.05$ using one-way ANOVA analysis followed by Tukey's test. Data are presented as mean ± SEM.

4.3.2 Tacrolimus immunosuppression by oral administration

Transplanted animals were treated with different dosages of orally-administrated tacrolimus for 8 consecutive weeks. At the end of the treatment tacrolimus treated groups

presented smaller weight and size compared to non-treated group (**Figure 9A**). Expression analysis of the whole embryo revealed that *il2* expression were significant higher in the group treated with 1.5 mg/Kg of tacrolimus compared to the non-treated group (Figure 9). In addition, *cd8* and *cd4* markers were also higher in this group compared to non-treated group and tacrolimus-treated groups (**Figure 9**).

Figure 9 – Growth parameters, qRT-PCR analysis of the transplanted embryos treated with tacrolimus



Source: YOSHINAGA, T.T, 2022

Legend: Growth parameters and qRT-PCR analysis of immune system makers *il2*, *cd8b*, and *cd4-1* at the end of tacrolimus treatment. The expression was normalized by the respective β-actin values. Different letters represent significantly difference for $p \leq 0.05$ using one-way ANOVA analysis followed by Tukey's test. Data are presented as mean ± SEM (n=6 in each group).

4.3.3. Donor-derived gametes production

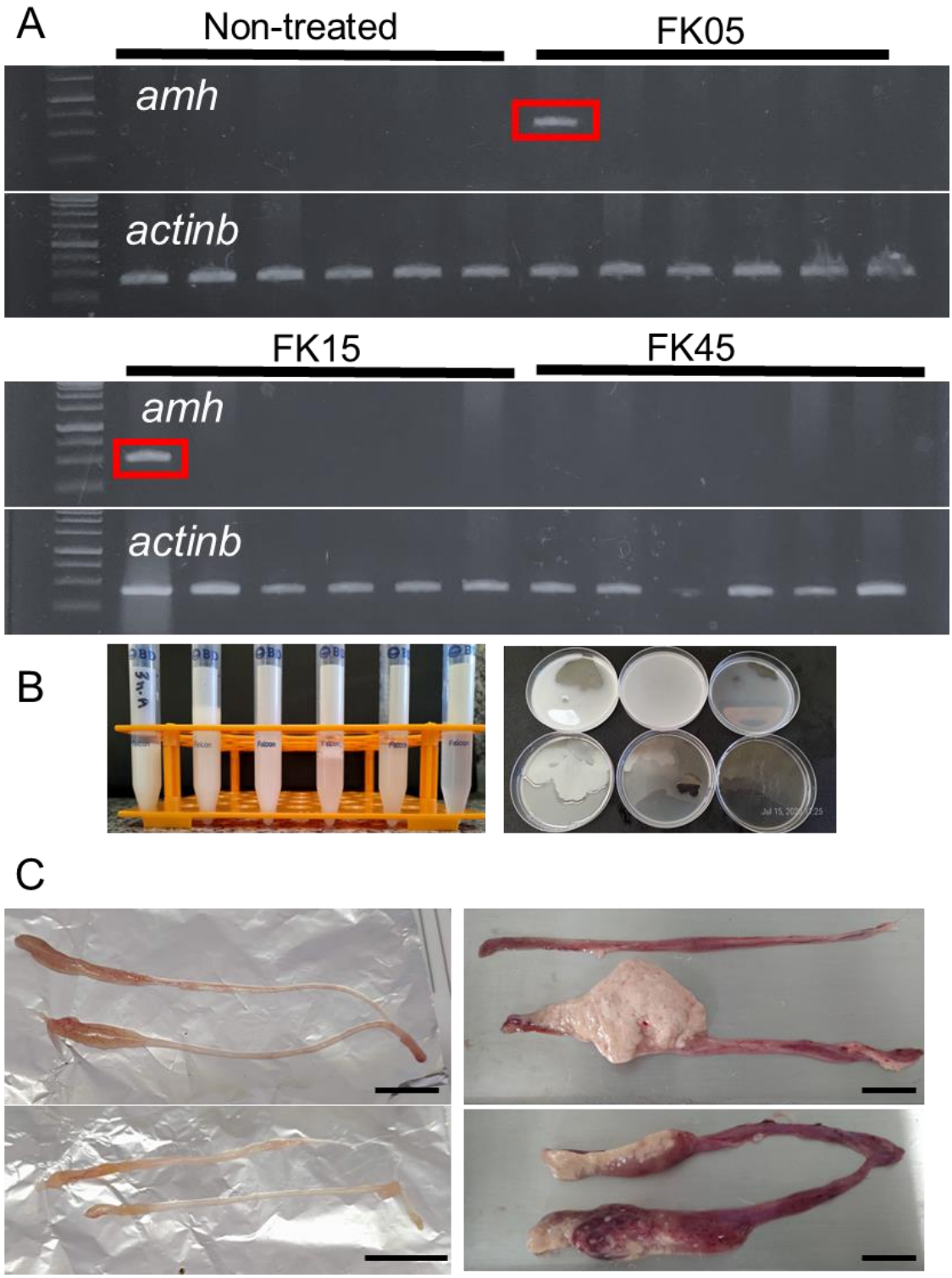
After the sexual maturation, gametes from transplanted animals were collected. None of transplanted females were able to produce eggs, even those treated with tacrolimus. On the other hand, males from all groups produced spermatozoa. The milt was collected, and PCR analysis revealed that one animal from tacrolimus 0.5mg/Kg and 1.5 mg/Kg were positive for Atlantic salmon anti-Mullerian hormone (*amh*) marker (**Figure 10A**). Some milt collected had a more viscous appearance, while other appeared more watered (**Figure 10B**). The spawned males and females are listed in **Table 2**.

Table 2 – Transplanted animals at sexual maturity

Group	Animals at the end of treatment	Adult animals	Spawned females	Spawned males
Non-treated	26	21	0	6
FK05	35	30	0	8
FK15	29	27	0	6
FK45	34	25	0	9

Macroscopic observation of the gonads from transplanted females revealed that ovaries were very thin with no ovarian follicles apparently. Some transplanted males presented normal testicular morphology, while other males presented testicular with irregular morphology (**Figure 10C**).

Figure 10 – PCR reaction of the spermatozoa produced by transplanted animals

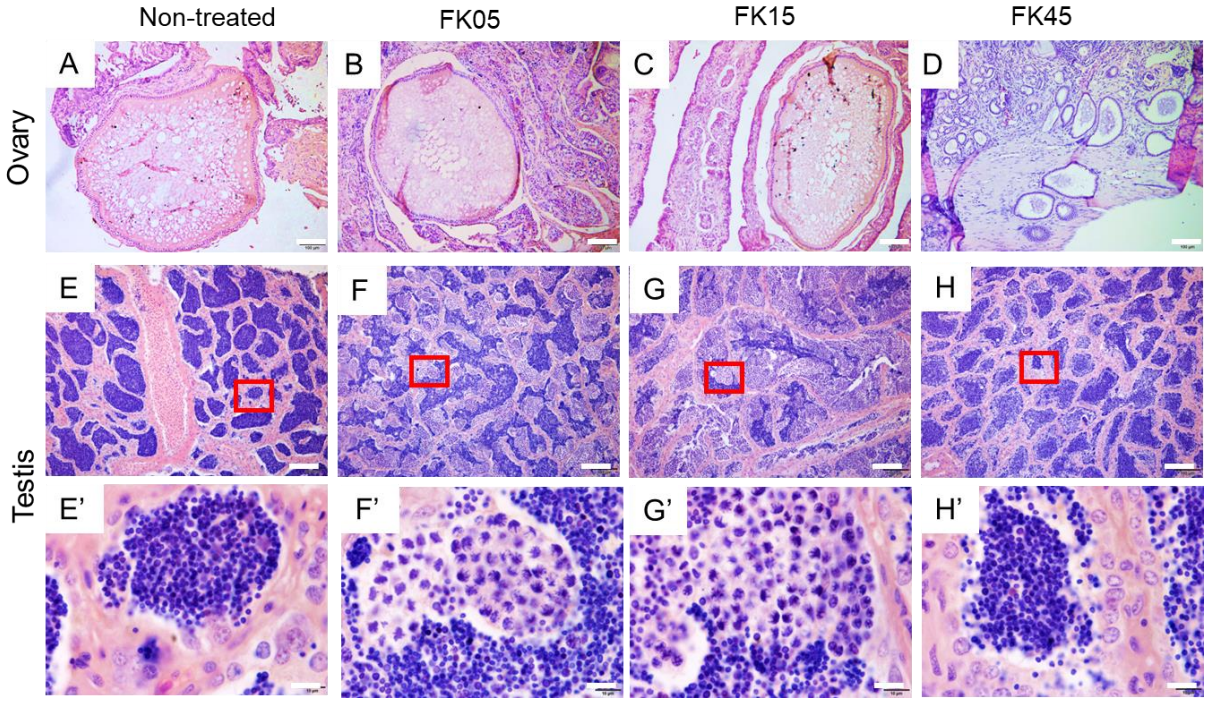


Source: YOSHINAGA, T.T, 2022

Legend: Conventional PCR from the milt produced by the transplanted animals after sexual maturation. (A) Animals positive for Atlantic salmon maker *amh* are highlighted with the respective *actinb* control. M: 1kb molecular marker. (B) Viscosity and color pattern from the milt produced by transplanted animals (C) Gonads from transplanted animals after sexual maturation. Scale bars indicate 2 cm.

Histological analysis conducted on the gonads from transplanted animals revealed that the ovaries contained few follicles in all groups (**Figure 11A-D**). On the other hand, testes from transplanted animals contained germ cells niches and germ cells during spermatogenesis in tacrolimus 0.5 and 1.5mg/Kg treated groups (**Figure 11E-H**). in the non-treated and in tacrolimus 4.5 mg/Kg group the testes did not present this conventional cystic structure with different germ cells stages. (**Figure 11-E-H**). No differences were observed for wight and size (**Figure 12**). The gonadosomatic index did not differ between treated and non-treated groups in both female and male animals (**Figure 12**).

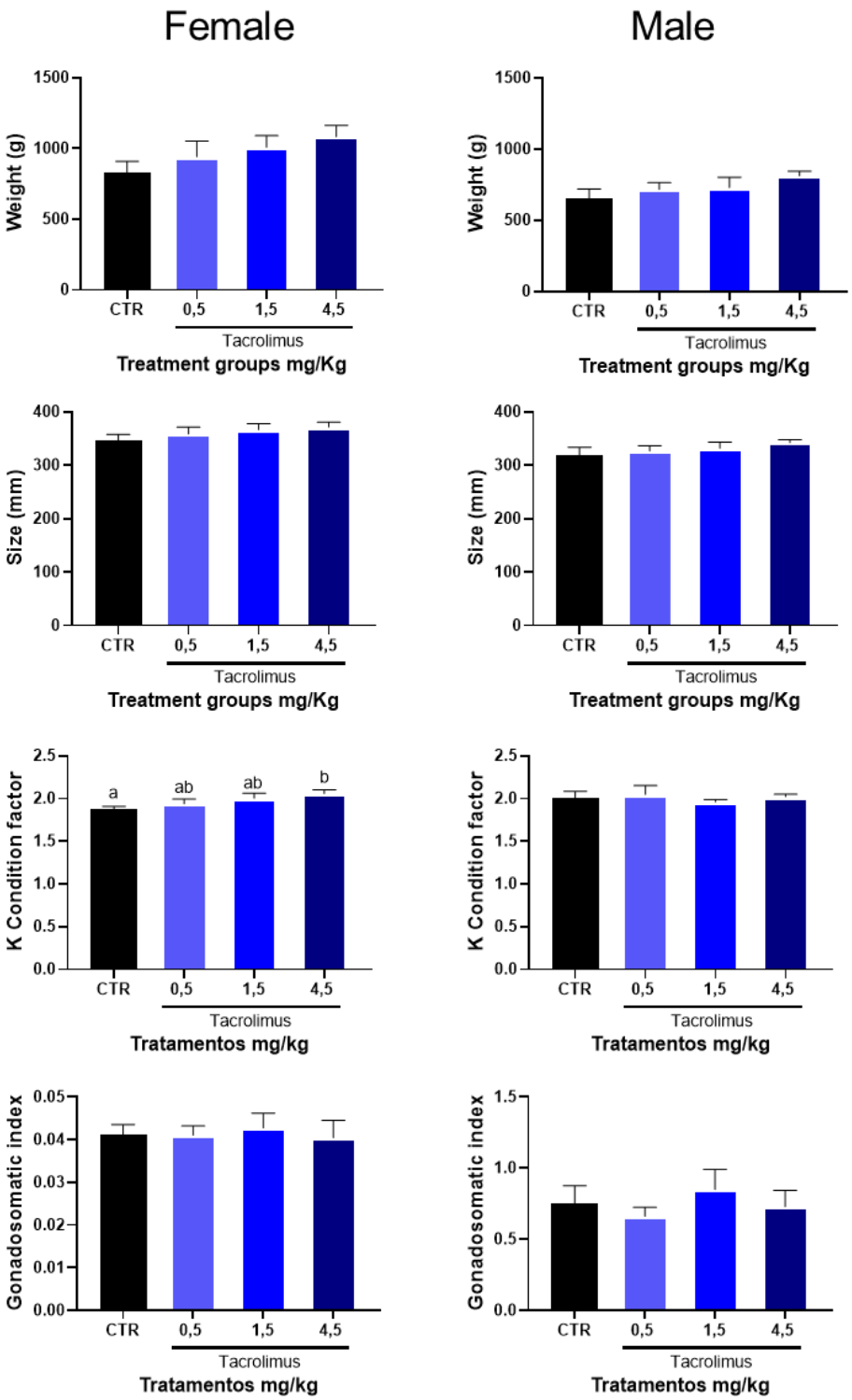
Figure 11 – Histological analysis of the gonads of the transplanted animals



Source: YOSHINAGA, T.T, 2022

Legend: Histological analysis of the gonads from transplanted animals. (A-D) Histological analysis of the ovary from transplanted females showing a sole oocyte follicle in non-treated, FK05, and FK15 groups. FK45 did not present oocyte follicle at the same stage. (E-F) Testicular of the transplanted animals showing FK05 and FK15 presenting clear spermatogenesis cyst, while non-treated and FK45 group different germ cells stages were not observed. Scale bars from A-B indicates 100 µm, E-H 100 µm, and E'-H' 10 µm.

Figure 12 - Growth parameters from transplanted female and male after sexual maturation



Source: YOSHINAGA, T.T, 2022

Legend: Growth parameters from transplanted female and male after sexual maturation. Different letters represent significantly difference for $p \leq 0.05$ using one-way ANOVA analysis followed by Tukey's test. Data are presented as mean ± SEM.

4.3.4. Generation of donor-derived offspring

The milt produce by the positive males for Atlantic salmon marker were used to fertilized rainbow trout eggs. Approximately 10g of rainbow trout eggs were fertilized using the milt from transplanted animals. Fertilized eggs reached the eyed-egg stage and hatched after 30 days incubation at 10°C. Most of the embryos died and few reached larval stage (**Figure 13**).

Figure 13 – Rainbow trout oocytes fertilized by Atlantic salmon milt produced by transplanted animals.

Source: YOSHINAGA, T.T, 2022

Legend: Rainbow trout oocytes fertilized by Atlantic salmon milt produced by transplanted animals. Progeny at eyed-egg stage, newly hatched embryos, and larvae. Scale bars indicate 1 cm.

4.4. Discussion

Surrogate broodstock technology has been proved to be an important approach for the aquaculture industry, and more importantly for the conservation of endangered fish species (JIN *et al.*, 2021). However, this biotechnology still faces limitations regarding the number of animals capable to produce donor-derived gametes, which could be related to immune rejection mechanisms on transplanted germ cells, if so, immunosuppressants may be considered as a potential alternative to overcome this mechanism of defense. In this study, we applied an orally tacrolimus treatment in rainbow trout transplanted with Atlantic salmon germ cells. Our results demonstrated that high expression of immunological markers was related to the reduced number of the germ cells in transplanted animals. In addition, the administration route used could not be able to sustain immunosuppression in the transplanted animals. Regardless, Atlantic salmon viable donor-derived spermatozoa were obtained and even produced progeny by the fertilization of rainbow trout eggs.

Immunosuppression in fish might be tricky to achieve, due to difficulties related to the administration of such substances in adult animals, even more in embryos. We speculated that

adding tacrolimus with to the feed could show some efficacy similarly to the administration of steroid hormones (ATAR; BEKCAN; DOGANKAYA, 2009). Expression analysis of immune related makers at the end of the treatment showed that 1.5 mg/Kg treated group were higher compared to the non-treated group, and other tacrolimus groups. Also, this group presented lower germ cell counts compared to non-treated group, which suggests an immune system interference on germ cell colonization of the host gonad. Although we expected a reduction on gene expression as reported by previous studies (XING *et al.*, 2017; YOSHINAGA *et al.*, 2021), the oral via used here may not be suitable to deliver the tacrolimus in embryos to achieve immunosuppressive effects. In addition, the effects of tacrolimus on embryos may not be the same as adult, due the young immune system (CASTRO *et al.*, 2015) or the lack of metabolic ability (COOK; BURNESS; WILSON, 2018). Thus, other ways of administration or the use other efficient methods such as nanoparticles or immersions in immunosuppressive solutions should be tested (KOOKARAM *et al.*, 2021; RATHER *et al.*, 2013). Despite, tacrolimus treated animals were smaller compared to non-treated group at the end of the treatment, immunosuppression during the larvae stage did not affect their normal development to adulthood and transplanted animals spawned normally.

Unfortunately, no oocyte was obtained by any transplanted female after sexual maturation. Macroscopic observation and histological analysis from the ovary of transplanted animals revealed that all groups presented fewer or no ovarian follicles and lower gonadosomatic index, which suggest that transplanted females were sterile. On the other hand, transplanted males from all groups produced spermatozoa and PCR analysis revealed two positive animals for Atlantic salmon marker from tacrolimus 0.5 and 1.5 mg/Kg treated groups. The produced milt was used to fertilize rainbow trout oocytes and was able to generate progeny, which demonstrated the viability of the spermatozoa produced by transplanted male as previously reported (HATTORI *et al.*, 2019). Although, we obtained donor-derived spermatozoa, the tacrolimus immunosuppression during the larvae stage did not affect or increased the number of animals producing donor-derived gametes as expected. Thus, at some point during gonads development, the transplanted germ cells may be eliminated until the transplanted animal reached sexual maturity, which contrasts with the fact of germ cells being protected by gonadal immune privilege mechanisms.

The gonads are considered immune privileged sites where the immune responses are regulated, especially the testes (LI; WANG; HAN, 2012; STEIN-STREILEIN; CASPI, 2014). However, this is not very well established for fish species and the immune system could attack

xenogeneic germ cells even inside the gonad after the immune system being fully functional (VALERO *et al.*, 2018). In addition to the immunological factor, the physiological compatibility may play a central role during transplanted germ cell gametogenesis. In case of donor-derived oocytes, oogenesis of transplanted germ cells may rely on other factors since oocytes receive several components from the host female (LUBZENS *et al.*, 2010). Although spermatogenesis is considered a simpler process, transplanted spermatogonial cells may need a correctly form to colonize the host testis, whereby transplanted cells could undergo gametogenesis and be able to release the spermatozoa. Some males from this study presented an abnormal testis morphology but did not present gametes in the spermatic duct. Thus, the lack of physiological competence by the transplanted germ cell or the host testis may also interfere in the spermatozoa and oocytes production by transplanted animals, as observed by other study using rainbow trout as host for different salmonids (MARINOVIĆ *et al.*, 2022). Thus, even transplantation conducted in an immunosuppressed host may not undergo gametogenesis without the right physiological stimuli.

Although the administration of tacrolimus used was not able to sustain immunosuppressive effects, the use of immunosuppressive treatment in transplant did not impair the production of donor-derived Atlantic salmon spermatozoa. In order to obtain successful immunosuppression, tacrolimus and other immunosuppressants can be administrated using alternative methods (KOOKARAM *et al.*, 2021; PATEL *et al.*, 2012). Also, to completely avoid the rejection of transplanted germ cells, immune-deficient lineages could be used as well (TANG *et al.*, 2014). However, such lineages are not established for many fishes and the immunosuppressive treatment may be the best option in these cases. Regarding to the concern related to susceptibility of the treated animal to diseases, we did not observe pathological signs in treated animals during the experiment. Nevertheless, most diseases can be controlled by management control or other medicaments in case of some infection in transplanted animals during immunosuppressive treatment (NASR-ELDAHAN *et al.*, 2021). In conclusion, improving the administration method with different immunosuppressants could be used to achieve immunosuppressive effects and improve surrogate broodstock technology in fish.

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Chapter 2

Tacrolimus and mycophenolate mofetil immunosuppressive therapy applied to subcutaneous testis allograft in rainbow trout (*Oncorhynchus mykiss*)

5. Tacrolimus and mycophenolate mofetil immunosuppressive therapy applied to inflammation in subcutaneous testis allograft in rainbow trout (*Oncorhynchus mykiss*)

5.1. Introduction

Teleost fishes consist of the primary vertebrate group which present a complex immune system (MAGADAN; SUNYER; BOUDINOT, 2015). Several mechanisms studied in mammals are also found in fish immunity and both have similarities in their mechanism of action (SUNYER, 2013), being immune rejection one of them. Tissue transplantation studies in fish started a long time ago using scale and skin grafts as an approach to identify immune rejection mechanisms from fish immunology (HILDEMANN, 1957, 1958). More recently, transplantation studies in fish can also be used to understand the ontogeny of immune system, cancer immunity, and for physiological studies using gonads transplantation (BHATTA *et al.*, 2012; TAYLOR; ZON, 2009). Interestingly, gonads transplantation studies can be applied as a reproduction biotechnology, since it is possible to obtain donor-derived gametes through the germ cell transplantation or by the testicular subcutaneous grafts (HAYASHI; SAKUMA; YOSHIZAKI, 2018; YOSHIZAKI; YAZAWA, 2019). Furthermore, testis grafts comprise a good approach to evaluate immune rejection mechanisms in such immune regulated tissue such as testis.

Although donor-derived gametes were produced by different host from different genus, family, and order in germ cell transplantation studies (JIN *et al.*, 2021), gonads grafts are only viable when performed between isogeneic lineages (CLOUD, 2003; NAGLER *et al.*, 2001), and functional spermatozoa from subcutaneous graft are only obtained in autografts (HAYASHI; SAKUMA; YOSHIZAKI, 2018). Clearly, transplanted gonads are immune rejected in allo- and xenotransplantation in the host organisms, and hence does not allow the grafted gonad to develop. In addition, the rejection of grafted tissues was also observed in skin and scale grafts in fish (NAKANISHI *et al.*, 2011; TODA *et al.*, 2009). In order to obtain long-term engraftments immune depleted or transgenic lineages are currently used (HESS *et al.*, 2013; TANG *et al.*, 2014). Alternatively, the immunosuppression of the host organism comprises an approach to obtain long-term engraftments as well as in organ transplantation in humans (VAN SANDWIJK; BEMELMAN; TEN BERGE, 2013).

Their use in fish remains poorly understood, as well as their application in transplantation in fish, but some studies suggests that their effects are similar as found in mammals. Cyclosporine and rapamycin inhibited leucocyte *in vitro* proliferation and reduce

leucocyte counts *in vivo* in flounder (*Paralichthys olivaceus*) (XING *et al.*, 2017). Cyclosporine and tacrolimus were also demonstrated to inhibit leucocyte *in vitro* proliferation and *il2* expression *in vitro* and *in vivo* in rainbow trout *Oncorhynchus mykiss*) (YOSHINAGA *et al.*, 2021). Furthermore, tacrolimus treatment was also demonstrated to delay spermatogonial immune rejection in testis subcutaneous allograft in rainbow trout (YOSHINAGA *et al.*, 2021).

Based on this information, we hypothesized that immunosuppression therapy could be applied in order to avoid immune rejection of testis grafts, and by the maintenance of the graft tissue, to obtain donor-derived gametes production. Thus, in this study we aimed to administrate a tacrolimus and mycophenolate mofetil mixture through emulsion solution in testis subcutaneous allograft model in rainbow trout and evaluate its effects.

5.2. Materials and methods

5.2.1. Fish rearing conditions and ethical statements

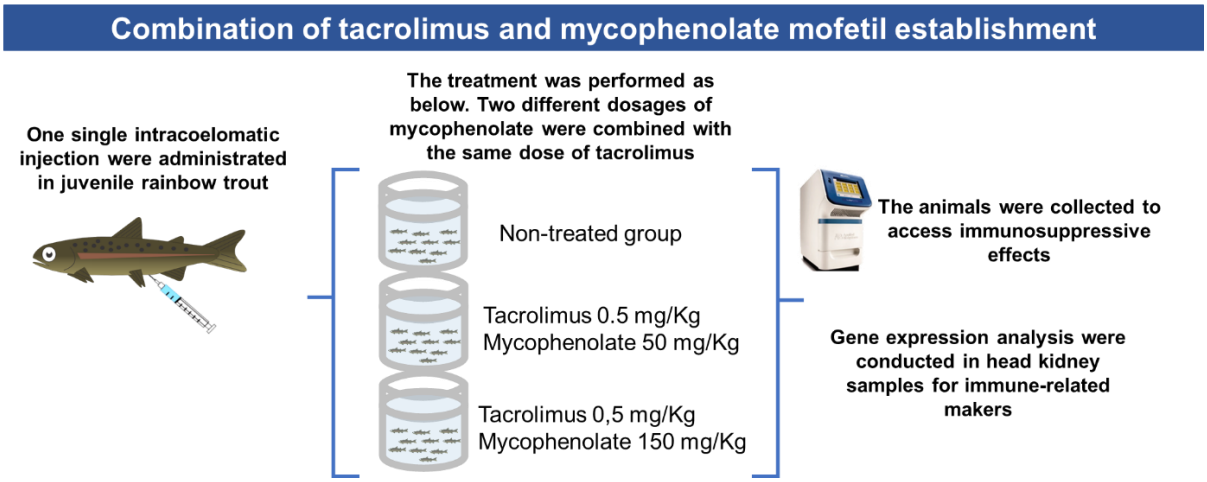
This study was conducted at the Salmonid Experimental Station at Campos do Jordão, UPD-CJ (Campos do Jordão, Brazil) and the School of Veterinary Medicine and Animal Science from the University of São Paulo, FMVZ-USP. All animals used were kept in $2m^3$ round tanks with constant water flow at 13 °C (+/- 2°C) under natural photoperiod conditions. Animals were fed commercial food twice a day during the entire experiment. All procedures were carried out in accordance with the guidelines for the care and use of laboratory animals from the School of Veterinary Medicine and Animal Science of University of São Paulo, certificate CEUA-6973220618.

5.2.2. Combination of tacrolimus and mycophenolate mofetil establishment

Accordingly with our previous study (YOSHINAGA *et al.*, 2021), we have established an optimal tacrolimus dosage also a suitable vehicle to administrate the immunosuppressants by intracoelomatic injection. Thus, in this work we combined the 0.5 mg/Kg tacrolimus dosage with the mycophenolate mofetil. Two different dosages of mycophenolate were tested based on the human posology, 50 mg/Kg, and 150 mg/Kg. Both immunosuppressive agents were dissolved in NaCl solution 0,7% and mixed with soil vegetal oil containing 1% of emulsifier PEG 30 Dipolyhydroxystearate (Cithrol DPHS or Arlacel P135, Croda, UK). The oil-water phase mixtures were vortexed for 30 minutes until acquiring a white-fluid solution. Thirty-six rainbow trouts of one-year old were divided in three groups containing twelve animals. The

first group receive a single injection of emulsion solution without any immunosuppressant. The second and third groups received a single injection of the combination of 0.5 mg/kg of tacrolimus and 50 and 150 mg/Kg of mycophenolate mofetil, respectively (**Figure 14**). The volume injected were calculated based on the weight of the animals to reach the correctly amount of immunosuppressants. Four animals from each group were collected three- and seven-days post injection to access immunosuppressive effects on immune system markers by qRT-PCR analysis.

Figure 14 – Scheme of the tacrolimus combination with mycophenolate

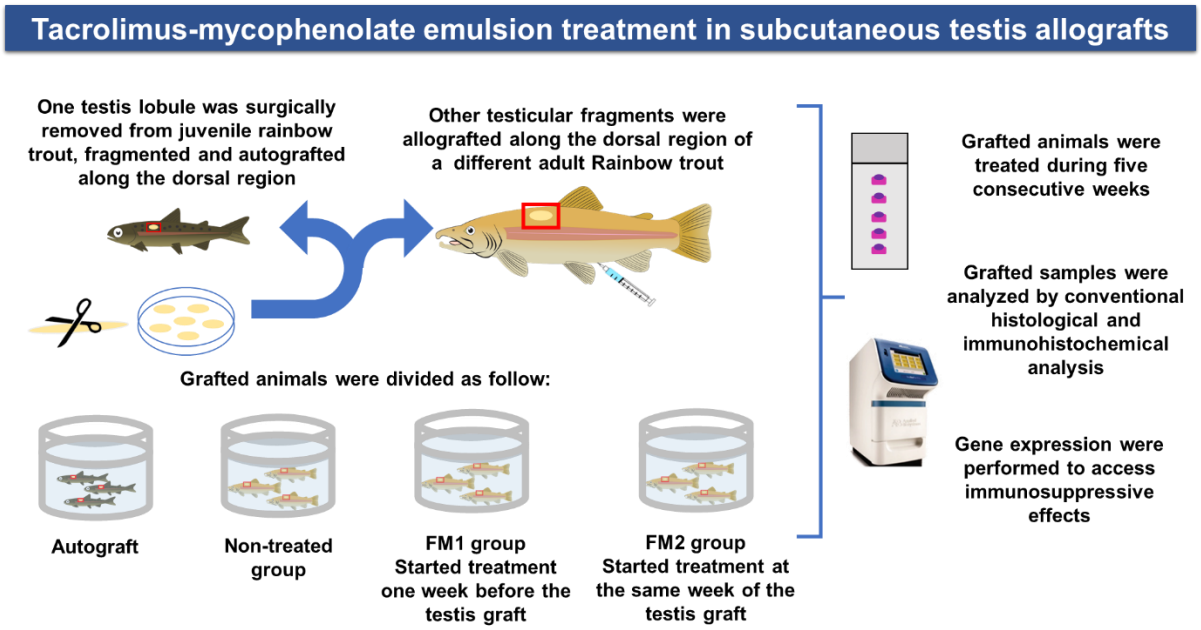


Source: YOSHINAGA, T.T, 2022

5.2.3. Testicular subcutaneous allograft in rainbow trout

Fish were sedated by immersion in benzocaine solution (0.1mg/L Sigma, USA) and placed in decubital position on a stand support. Then, a 4 cm mid-ventral incision was made with a scalpel and the left testis was removed from a one-year-old juvenile rainbow trout. The incision was closed with four sutures using silk thread (3-0) and treated with iodine solution. The isolated testis was fragmented in 4 equal parts, which was grafted along the back of the donor rainbow trout. One cm below the dorsal fin, a small incision was made with a scalpel and the skin, and the muscle were separated using a forceps creating a small pocket towards to the head. One part from the testis fragments were carefully inserted into this pocket. This procedure was performed in the donor group (autograft) right after the abdominal surgery to remove the testis lobe. The remaining fragments were then grafted as the same way in two-year-old rainbow trouts (allografts) (**Figure 15**). After the procedures fish were returned to the tanks for recovery. All the incisions spontaneously healed after about four weeks.

Figure 15 – Scheme of the subcutaneous testicular allograft treated with tacrolimus and mycophenolate emulsion



Source: YOSHINAGA, T.T, 2022

5.2.4. Immunosuppressive treatment using emulsion-containing immunosuppressants

One of the treated allograft groups started the treatment with emulsions-containing tacrolimus and mycophenolate mofetil one week before the testis graft (FM1 group). The day of the second dosage was also the day which the testis graft was performed in all fishes. Other fish group start the treatment with the same dosage at the same day of the testis graft (FM2 group), and one allografted group remining non-treated. Allografted fishes received a weekly treatment along 5 consecutive weeks with the emulsion containing immunosuppressants, while the non-treated group receive only the vehicle injections as the previous tested. Testis grafts samples were collected exactly five weeks post-transplantation for histological and gene expression analysis for testicular cell markers. Head kidney samples were collected to access the immunosuppression in the grafted fishes treated with the immunosuppressive therapy.

5.2.5. Conventional histological and immunohistochemical analysis

Grafts samples were fixed with Bouin's solution at 4 °C for 24 hours. Then, embedded in paraffin and sliced in 5-µm thickness sections. Graft sections were stained with hematoxylin and eosin (HE) for morphological observation of the testicular tissue.

Immunohistochemical (IHC) procedure was performed as followed. Graft sections were deparaffined, re-hydrated, and submitted to antigen retrieval process using citrate buffer

solution with 0.5% Tween 20 (pH 6.0) in microwave. The sections were heated until boil, then maintained in this state for 7 minutes. Endogenous peroxidase activity was blocked using 3% H_2O_2 in 0.01M PBS at room temperature for 30 minutes in the dark., Then the sections were rinsed in 0.01M PBS containing 0.5% Tween 20 and blocked with horse serum from ImmPRESS® Universal Reagent (Vector Laboratories) for 30 minutes. The sections were incubated with the primary antibody #189 (HAYASHI *et al.*, 2019), to detect undifferentiated spermatogonia using 1:250 dilution, during 16 hours at 4 °C. After the primary antibody incubation, the sections were rinsed in 0.01M PBS and incubated with ImmPRESS® Universal Secondary Antibody (Vector Laboratories) for 30 minutes at room temperature. Then, the sections were rinsed in 0.01M PBS, incubated with ImmPACT® DAB Substrate, Peroxidase (HRP) (Vector Laboratories), counter-stained with hematoxylin and mounted. Positive control was performed using rainbow trout testicular tissue and negative control was performed without the primary antibody as demonstrated previous reported (BUTZGE *et al.*, 2021).

5.2.6. RNA extraction, cDNA synthesis, and qRT-PCR analysis

Tissue samples were fixed in RNA later (Invitrogen, USA) during 12 hours at room temperature and stored at -80°C until further analysis. The total RNA was extracted using TRIzol Reagent (Invitrogen, USA) with the L-beader 6 cell disruptor tissue homogenizer (Loccus Biotechnology, Brazil) with zircon beads. RNA concentration was measured using the NanoDrop™ 2000 Spectrophotometer. A total of 1 µg of RNA was treated with DNase I amplification grade (Invitrogen, USA), and first strand cDNA was synthesized by SuperScript III (Invitrogen, USA) with $OligoDT_{12\text{-}18}$ primer (Invitrogen, USA). All procedures were performed following manufacturer's instructions.

The expression analyses by qRT-PCR were performed using SYBR Green Master Mix (Applied Biosystems) in the StepOnePlus™ Real-time PCR system (Applied Biosystems) using 10 µl reaction volume. The primer sequences, amplicon size, and GenBank access numbers are listed in **Table 3**. The two-step qPCR condition was as follow: 40 cycles of 95 °C for 60 s, and 60 °C using the StepOne Software 2.3 version. The transcript abundance was quantified by the Standard Curve Method with four dilution points and normalized using the respective *β-actin* values.

Table 3 – Primers sequences and amplicon size

Gene name	Primer sequence (5' → 3')	Amplicon size in bp	Genbank accession number
il2	Fw: CATGTCCAGATTCAGTCTTCTATACACC Rv: GAAGTGTCCGTTGTGCTGTTCTC	211	NM_001164065.2
ifnγ	Fw: CCGTACACCGATTGAGGACT Rv: GCGGCATTACTCCATCCTAA	256	NM_001124620.1
cd8b	Fw: GGTTGTACTCCTGTATGCTCC AGA3 Rv: AGAAGAACTCCTGGCCTCCACAAC	64	NM_001124008.1
cd4-1	Fw: CGAGAGACGATAGATCCAGAGTGGAAAG Rv: TGTGACTGAGGTACTTGTTTGTGGCATGA	150	NM_001124539.1
impdh2	Fw: GGTCATTGGAGGCAATGTGG Rv: TGCCAACACTTCCTGTGTGAT	121	XM_021566530.1
vasa	Fw: CTACTGGAGGTCCTGAAGACTAC Rv: AGTATTAACCTTCTCCCGACACA	126	NM_001124193.1
txdnc6	Fw: AGCAAAGAAAACGCTATCGCAGAA Rv: CATGGCGTGCTGGACATTGG	156	XM_021622360.1
amh	Fw: GGGAATAACCATGCTATCCTGCTT Rv: CTCCACCACCTTGAGGTCCTCATAGT	108	XM_021603833.2
actinb	Fw: GCACTGGTTGTTGACAACGGA Rv: AACCATCACTCCCTGATGCCT	117	NM_001124235.1

5.2.7 Statistical analysis

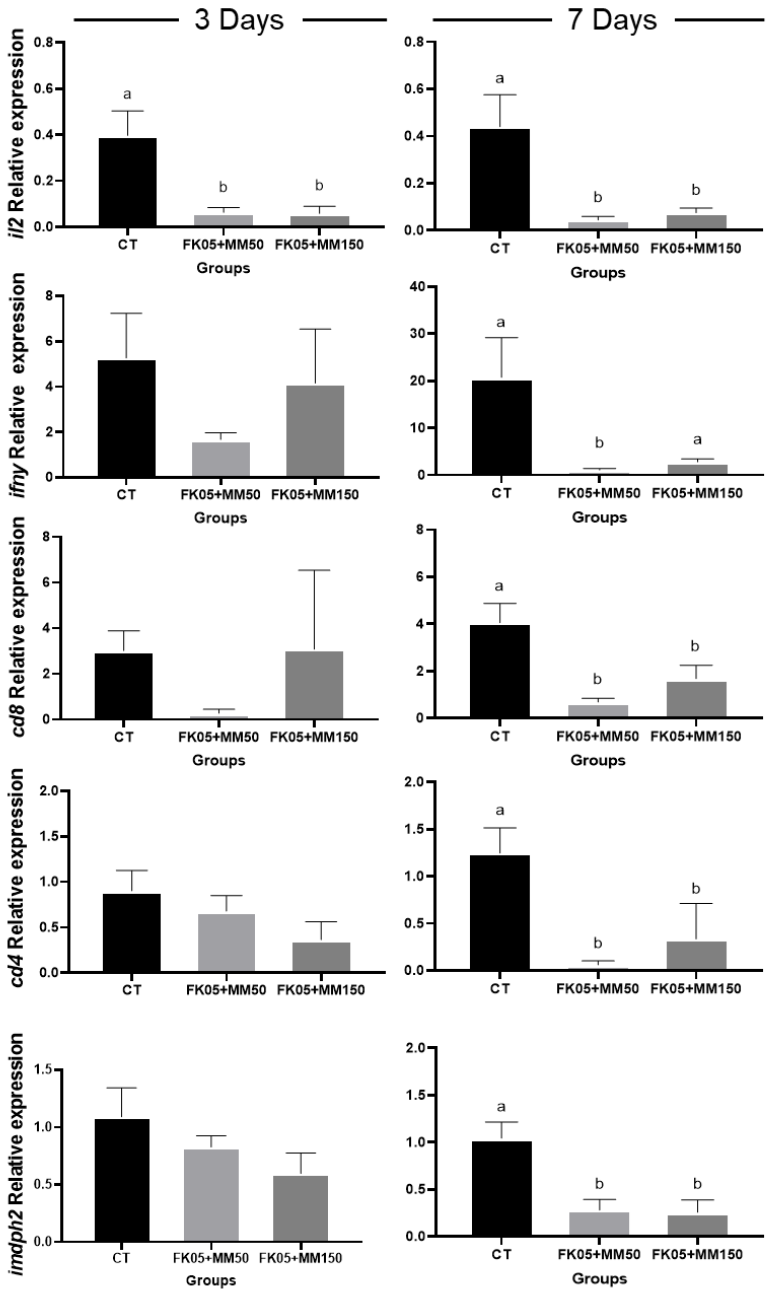
All data were analyzed in GraphPad Prism 9.4 (GraphPad Software, USA) using One-way ANOVA, followed by Tukey's multiple comparison test and Pearson's Correlation test. The level of significance was considered for $p \leq 0.05$. All data were presented as mean ±S.E.M.

5.3. Results

5.3.1. Immunosuppressive effects from the combination of tacrolimus with different mycophenolate mofetil dosages

In this first trial, tacrolimus was mixed with two different dosages of mycophenolate mofetil. Three days post-injection, the animals treated with both combinations presented an inhibition of the *il2* expression that was also observed seven days post injection (**Figure 16**). Another cytokine analyzed was *ifnγ*, which did not show altered expression three days post injection, but a significant inhibition after seven days. T cell markers *cd8* and *cd4* also did not present inhibition three days post injection but showed lower expression seven days post injection. This same pattern was also observed for inosine monophosphate dyhidrogenase2 (*impdh2*) (**Figure 16**).

Figure 16 - Expression analysis from the combination of tacrolimus with different dosages of mycophenolate mofetil.

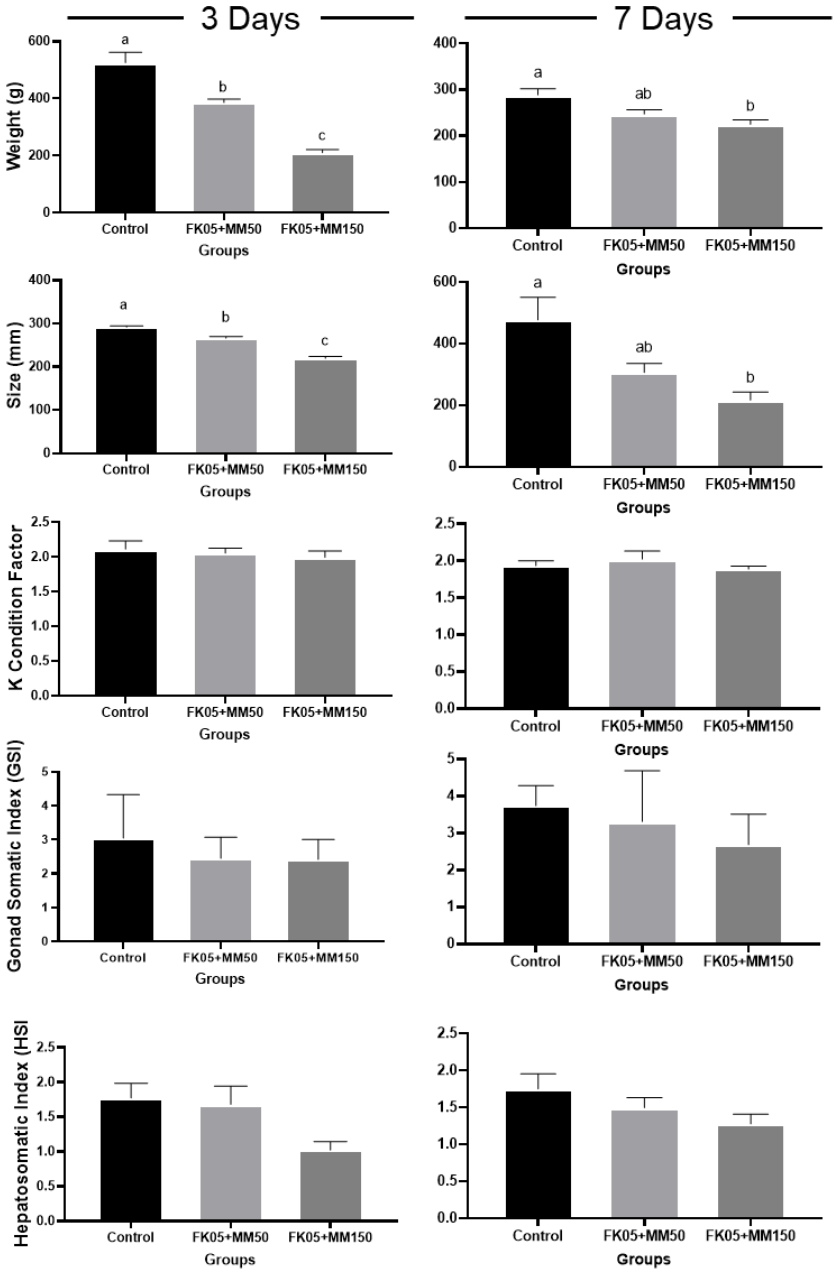


Source: YOSHINAGA, T.T, 2022

Legend: qRT-PCR analysis of the immune system markers *il2*, *ifny*, *cd8b*, *cd4-1*, and *imdph2* three- and seven-days post injection of tacrolimus-mycophenolate immunosuppressive therapy. Reduced expression was observed in both combinations seven days post one single injection. The expression was normalized by the respective *β*-actin values. Different letters represent significantly difference for $p \leq 0.05$ using one-way ANOVA analysis followed by Tukey's test. Data are presented as mean ± SEM (n=6 in each group).

The animals used in this trail were different in size and weight because they were chosen specifically for this pilot. As the emulsion volume injected was based on the weight of the animals, this difference should not interfere in the results. In addition, other parameters were not different such as the K condition factor, gonad somatic index, and hepatosomatic index (Figure 17).

Figure 17 – Growth parameters from the tacrolimus-mycophenolate combination



Source: YOSHINAGA, T.T, 2022

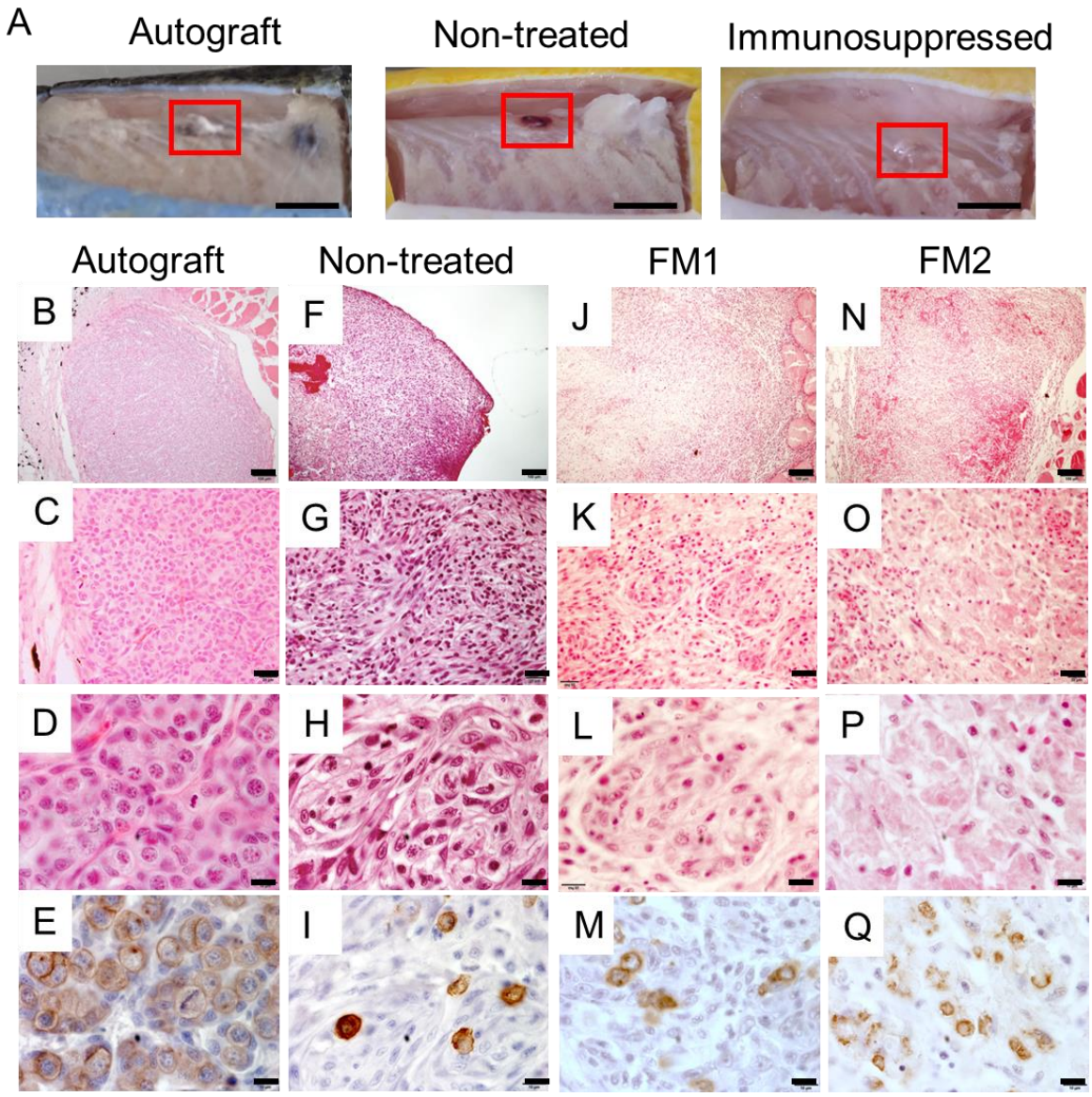
Legend: Different letters represent significantly difference for $p \leq 0.05$ One-way ANOVA analysis followed by Tukey's test. Data are presented as mean ± SEM.

5.3.2. Testicular subcutaneous graft followed by immunosuppressive therapy

Based on previous studies (YOSHINAGA *et al.*, 2021), histological analyses were carried out 5 weeks post-graft. The skin was separated from the muscle to access the graft region. The grafted testis appeared clear with a withe-pinkish color aspect in the donor group and in the treated group without signs of inflammation (**Figure 18A**), while in the non-treated group it was surrounded by a reddish color in the grafted area (**Figure 18A**).

A microscopical analysis from the graft revealed a normal testicular structure in the autografted group containing germ cell cysts similar to those found in normal testis. Immunohistochemical analysis demonstrated that the observed cells were indeed spermatogonial cells (**Figure 18B-E**). On the other hand, non-treated group (**Figure 18F**-I) and both immunosuppressed groups (FM1 and FM2) (**Figure 18J-Q**) did not present the same structures as observed in the autograft group. High quantities of mononuclear cells were present inside the allografted testis from the non-treated, FM1, and FM2 groups, with unclear cell boundaries, and hyalin deposits spread along the tissue (**Figure 18F-Q**). Immunohistochemical analysis conducted in these samples revealed few germ cells marked, most of the marked cells did not appear as the testicular tissue from autografted group.

Figure 18 – Macroscopical and histological analysis of testicular allografts treated with tacrolimus and mycophenolate



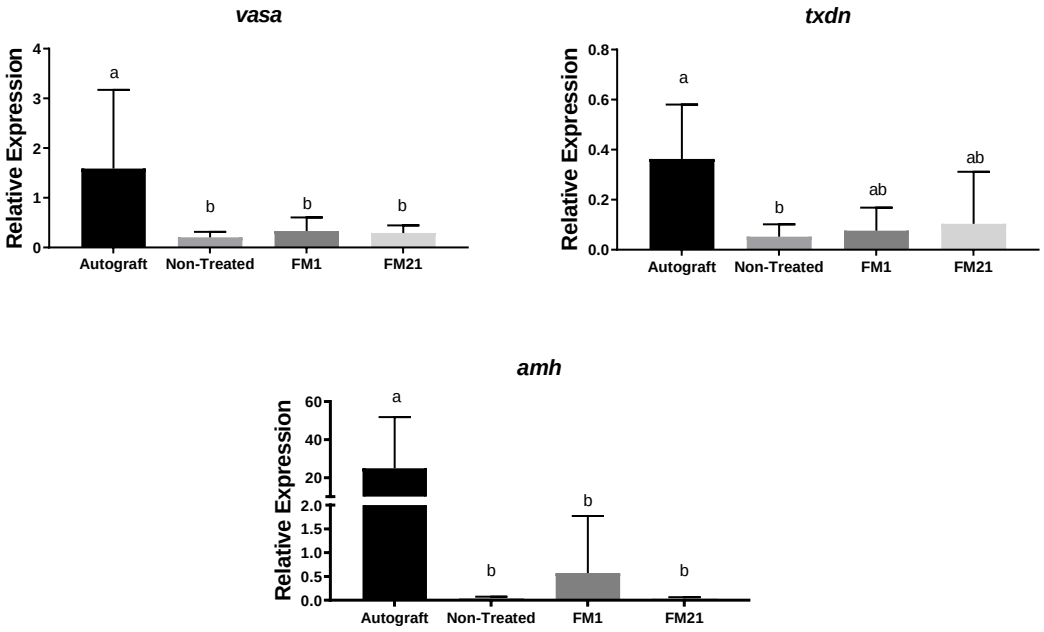
Source: YOSHINAGA, T.T, 2022

Legend: (A) Subcutaneous testis graft evidencing the detached skin from the muscle. Histological analysis evidencing the grafted testicular tissue in autograft (B, C, and D), non-treated group (F, G, and H), FM1 (J, K, and L), and FM2 (N, O, and P). (E, I, M, and Q) Immunohistochemistry revealed the presence of germ cells in the grafted testicular tissue of the autograft group, while in non-treated group, FM1 and FM2 groups, labelling was weakly observed, and the germ cells did not show normal morphology. Scale bars from B, F, J, and N indicates 100 μm, from C, G, K, and O indicates 50 μm, from D, H, L, and P indicates 10 μm, and from E, I, M and Q indicates 10 μm.

Expression analysis from the graft region for germ cell markers revealed that autograft group presented a higher *vasa* expression compared to the other groups, while *txdn* was

significant higher in relation to non-treated group. The somatic cell maker *amh* was also significantly higher in autograft group compared to other groups (**Figure 19**).

Figure 19 – Expression analysis of testicular germ and somatic cells markers

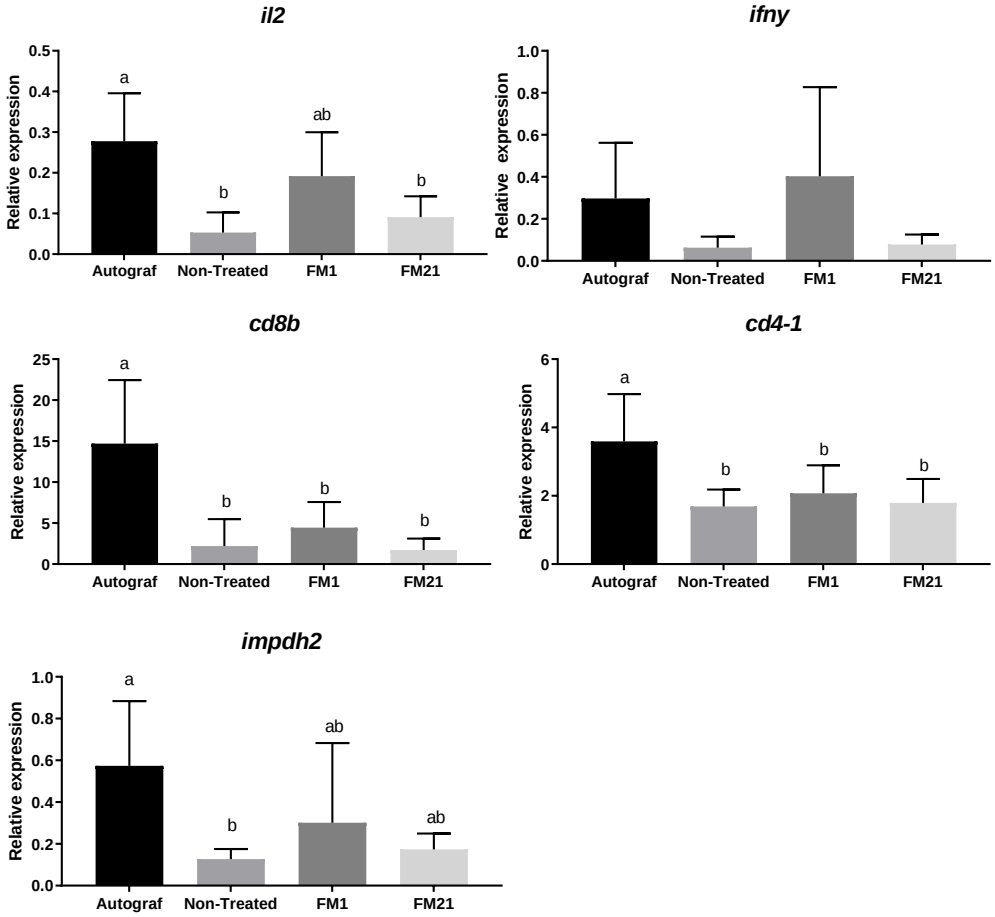


Source: YOSHINAGA, T.T, 2022

Legend: qRT-PCR analysis of the germ cell makers *vasa*, *txdn*, and the Sertoli cells marker *amh* in the testicular tissue grafted. The expression was normalized by the respective *β*-actin values Different letters represent significantly difference for $p \leq 0.05$ One-way ANOVA analysis followed by Tukey's test. Data are presented as mean ± SEM. (n=6 in each group).

Gene expression from head kidney samples was also performed in the groups to evaluate the long-term immunosuppression treatment. Intriguingly, autograft presented an *il2* expression higher than non-treated group and FM2. The *ifny* expression was not affected by the immunosuppressive therapy. The T cell markers *cd8* and *cd4* were significant smaller in non-treated, FM1 and FM2 groups compared to the autograft group. *imdph2* expression was also higher in the autograft group compared to the non-treated and FM2 group

Figure 20 – Expression analysis of immune-related marker in head kidney of allografted animals



Source: YOSHINAGA, T.T, 2022

Legend: qRT-PCR analysis of the immune system markers *il2*, *ifny*, *cd8b*, *cd4-1*, and *imdph2* in the head kidney of the grafted animals treated with tacrolimus-mycophenolate immunosuppressive therapy. The expression was normalized by the respective *β*-actin values. Different letters represent significantly difference for $p \leq 0.05$ One-way ANOVA analysis followed by Tukey's test. Data are presented as mean ± SEM. (n=6 in each group).

5.3.3 Histological analysis of the testis from the host animals

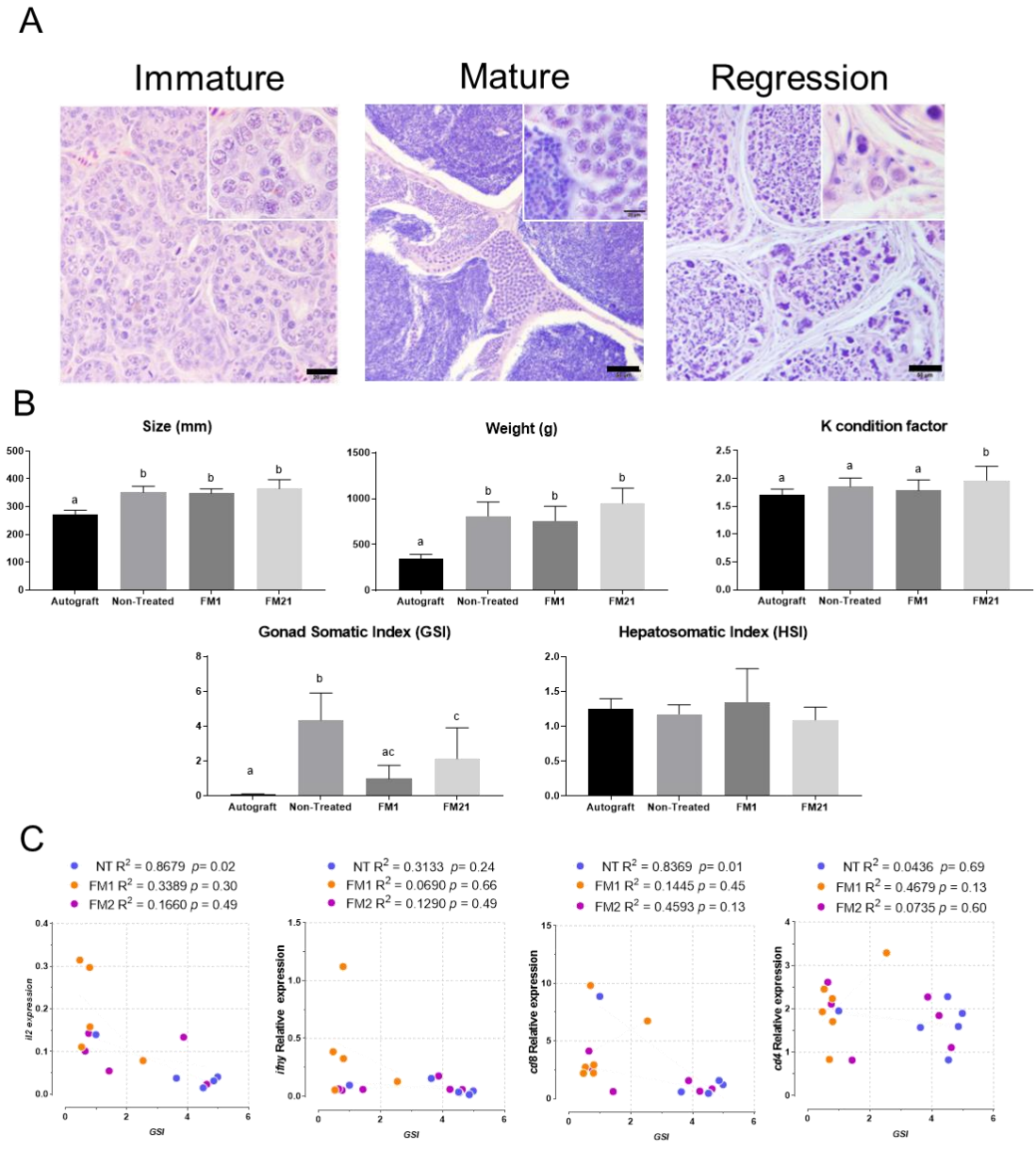
Histological analysis of the testis from the grafted animals revealed the maturation stage of the gonad (**Table 4**, **Figure 21A**). Autografted animals presented a gonad completely full of spermatogonial cells or in an initial stage of spermatogenesis. In both immunosuppressed groups and the non-treated group, some animals presented a testis in spermatogenesis while some animals were in a regression state, with a high quantity of cells with condensed nuclei, being spermatogonial cells the only germ cell observed.

Furthermore, the gonad somatic index (GSI) was measured from the grafted animals (**Figure 21B**). Autografted group presented the smallest value, while the non-treated group presented the highest. The immunosuppressed group 1 value did not differ significatively from the autograft group, but it was significant smaller than non-treated group. The immunosuppressed group 2 presented a smaller GSI compared to non-treated group, but higher than both autograft and immunosuppressed group 1. The high GSI demonstrated a high correlation with the *il2* and *cd8* gene expression in non-treated group. This relation was not observed for both immunosuppressed groups (**Figure 21C**).

Table 4 – Gonadal stage from the grafted animals

Group	Spermatogonial	Spermatogenesis	Regression
Autograft	6	2	0
Non-treated	0	7	1
FM1	0	2	4
FM2	0	6	2

Figure 21 – Histological analysis of the gonads of the autograft and allografted animals



Source: YOSHINAGA, T.T, 2022

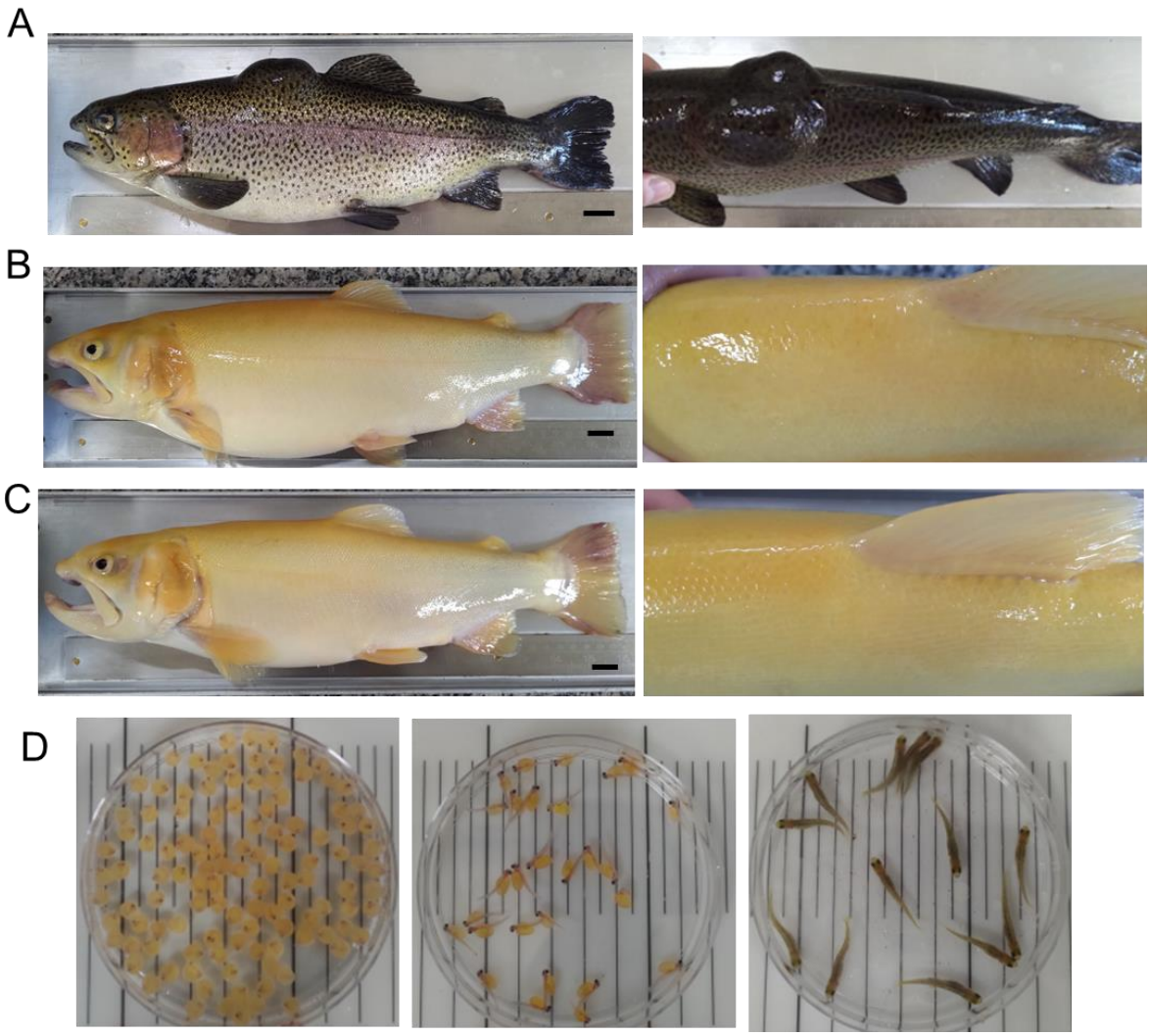
Legend: (A) Histological analysis from testis from the grafted animals. (B) Growth parameters, condition factor, gonadosomatic, and hepatosomatic index. Data are presented as mean ± SEM (C) Correlation analysis (R^2) of immune related gene expression with gonadosomatic index.

5.3.4. Donor-derived gametes production

The remained grafted animals were maintained to access donor-derived gametes production. In the autografted group, three animals presented the grafted testis in the grafted region and produced milt in the grafted region (**Figure 22**). The spermatozoa were collected to fertilized eggs from rainbow trout female. No animals from immunosuppressed group presented

growth in the grafted region neither from the non-treated group. The grafted region did not appear as the autografted group (**Figure 22**). The animals were maintained for 6 to 9 months after the testis graft. Egg fertilization rate and eye-egg stage are listed in **Table 5.**

Figure 22 – Autografted and allografted rainbow trout three months after testicular graft



Source: YOSHINAGA, T.T, 2022

Legend: (A) Autograft group presented a protuberance on the back three months post-graft, while (B) non-treated and (C) immunosuppressed animals were normal. Scale bars indicate 2cm (D) fertilized eggs at eyed-egg, newly hatched, and larval stage from the milt collected from the Autograft group

Table 5 – Fertilization rates from milt produced by testicular autograft

	Number of fertilized oocytes	Percentage Eyed egg stage	Percentage of hatchling
	249	79.5%	85%
	247	82.5%	90%
	261	69.7%	82%
Mean ±SEM	252 ± 5.7	77.2 ± 5	85 ± 2.8

5.4. Discussion

Gonad transplantation in fish has been performed for a long time (CLOUD, 2003; NAGLER *et al.*, 2001) and after the development of the surrogate broodstock technique, it also started to be used as reproductive biotechnology (HAYASHI; SAKUMA; YOSHIZAKI, 2018; TAKEUCHI; YOSHIZAKI; TAKEUCHI, 2004). Nevertheless, allo and xenogeneic testicular grafts are rejected by the host immune system which does not allow the production of donor-derived gametes (HAYASHI; SAKUMA; YOSHIZAKI, 2018; YOSHINAGA *et al.*, 2021). Thus, we hypostatized that an immunosuppressive therapy could avoid immune rejection in testicular subcutaneous allografts. In this study, a combination of tacrolimus and mycophenolate demonstrated immunosuppressive effects for one week in rainbow trout after a single injection. A consecutive treatment during five weeks in subcutaneous testis allografted animals was able to reduce acute inflammation. Nevertheless, the allografted testis presented signs of immune rejection in treated animals and analysis on the host gonads revealed that the testis was in regression state, which may reduce the host ability to maintain the grafted testis. Thus, both immunosuppression and a fully mature male host are required in order to maintain allografted testis.

The combination of tacrolimus with mycophenolate mofetil is a common treatment used for organ transplantation in human (MORALES; DOMÍNGUEZ-GIL, 2006; TUSTUMI *et al.*, 2021) and more effective to avoid immune rejection (HALLECK *et al.*, 2013). We have established a suitable dosage of tacrolimus (YOSHINAGA *et al.*, 2021) and thus, we just established the dosages of mycophenolate in emulsions solutions. Notably, the effects of 0.5mg/kg of tacrolimus with 50mg/Kg of mycophenolate on cytokine expression were more powerful than our previous work and fortunately lasted for at least seven days after a single

injection. However, a five-week consecutive treatment with weekly injection in allografted animals did not demonstrate the same effects. The non-treated group presented lowest expression values compared to the immunosuppressed groups and the autograft group, which was an intriguing result. During the graft experiment, we have noticed that the non-treated group presented a higher GSI compared to the immunosuppressed groups, which correlates with the lower expression of the few analyzed genes and may contribute to reduce immune system function. Reproductive hormones were demonstrated to modulate immune responses and alter gene expression in fish, and hence may interfere immune system function of the non-treated animals (BUPP; JORGENSEN, 2018; CHAVES-POZO; GARCÍA-AYALA; CABAS, 2018; VALERO *et al.*, 2018). In addition, the long-term exposure to the emulsion solutions may have different effects in contrast to the single dosage used in the previously experiment (LI *et al.*, 2020; TAFALLA; BØGWALD; DALMO, 2013). Regardless the effects on the gene expression, the immunosuppressive treatment indeed reduced the acute inflammation of the allografted testis in the treated animals.

At the end of the treatment, histological analysis revealed the graft condition and immunohistochemistry evidenced the presence of germ cells. Autograft group presented a normal testicular tissue, which was not observed in non-treated group and in both immunosuppressed groups. The grafts were severe impacted, presenting infiltrated leucocytes and tissue damage signs. Moreover, the non-treated group presented germ cell maker as observed in immunosuppressed groups, which was not observed in previous experiments (YOSHINAGA *et al.*, 2021). Although the immunosuppressive treatment may contribute for the graft survival, the testicular maintenance may rely on other factors beyond the immunosuppression itself. Reproductive signals are required to the testicular development (SCHULZ *et al.*, 2010; URIBE; GRIER; MEJÍA-ROA, 2014) and may be essential for the graft testis maintenance as well. The lack of such signals influences the testis graft maintenance, once the grafted testis will follow the host testis development (HAYASHI; SAKUMA; YOSHIZAKI, 2018). Thus, the immunosuppression of the host with the reproduction stimuli are essential to the testis graft maintenance in the allografted host. On the other hand, autografts did not required immunosuppression to produce functional gametes through subcutaneous testis grafts.

After few months, the autografted animals started to show a protuberance on the graft region. The grafted testis produced viable spermatozoa that was able to fertilize rainbow trout oocytes as demonstrated by a previous study (HAYASHI; SAKUMA; YOSHIZAKI, 2018). Although we did not expect immune rejection in autografts, not all the animals were able to

maintain the testicular graft and produce viable gametes. The condition of testis may need also be appropriate to be grafted, as well to isolating germ cells for transplantation studies (OKUTSU *et al.*, 2006b). Testicular tissue containing high quantity of differentiated cells will probably be easily recognized by the immune system, since differentiated germ cells such as spermatocytes, spermatids, and spermatozoa lack cell plasticity to survive in graft microenvironment (KAUR; MITAL; DUFOUR, 2013). Therefore, highly immature testis has more chances to survive even in allograft conditions compared to testis in early stages of spermatogenesis.

In summary, tacrolimus and mycophenolate mofetil administration through emulsion injections inhibited cytokine expression in rainbow trout. Also, this combination was able to reduce acute inflammation in testicular subcutaneous allograft. Nevertheless, the immunosuppression itself was not able to avoid the allografted testis immune rejection. Testicular graft maintenance depends on both immunosuppression and physiological conditions of the host. Thus, grafting an immature testis in a fully mature adult animal combined with the immunosuppressive therapy developed in this study, the production of allogeneic spermatozoa might be achieved in subcutaneous testis allografts. Furthermore, subcutaneous testis allograft allows the production of only donor-derived gametes and does not require sophisticated equipment as germ cell transplantation approaches. This technology can also be applied for the reproduction of commercial and endangered species, and to the study immune rejection mechanisms on immune privilege organs such as the testis.

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Chapter 3

Effects of immunosuppressive treatment on male rainbow trout (*Oncorhynchus mykiss*) reproduction

6. Effects of immunosuppressive treatment on male rainbow trout (*Oncorhynchus mykiss*) reproduction

6.1. Introduction

The immunosuppressants includes substances usually used to suppress the immune system in organ transplantation and their discovery had changed transplantation success, improving the patient survival and the longevity of the transplanted organ (HALLECK *et al.*, 2013). Although the effects of such substances on fish immunology remains unexplored, recent studies demonstrated immunosuppressive effects of cyclosporine, tacrolimus, and rapamycin on the inhibition of leucocytes proliferation *in vitro*, reducing leucocytes counts in peripheral blood, and inhibiting gene expression (CAO *et al.*, 2021; XING *et al.*, 2017).

Recently it was suggested that reproductive biotechnologies such as germ cell transplantation and testicular grafts may benefit from immunosuppression of the host in order to avoid immune rejection against the transplanted cells and grafted testis (YOSHINAGA *et al.*, 2021). Considering that immune rejection is a problem inherent to any transplantation procedure, donor transplanted cells and grafted testis are subjected to the action of the host immune system. However, although the immunosuppressive effect on fish immune system may be similar as found in mammals, there are concerns related to the susceptibility of treated animals to diseases as well as their side effects on fish physiology.

Studies on immunosuppressants side effects demonstrated that cyclosporine and mycophenolate mofetil disrupt blood vessels formation and pericardial edema in zebrafish embryos (JIANG *et al.*, 2016; PANDEY *et al.*, 2015). In addition, tacrolimus was demonstrated to cause abnormalities of amputated fins in adult zebrafish (KUJAWSKI *et al.*, 2014), while azathioprine administration in adult Nile tilapia increases spleen and pronephros size (GOGAL *et al.*, 1999). Although side effects on fish reproduction are unknown, several studies report their effects on male fertility (DROBNIS; NANGIA, 2017) and the prolongated use of immunosuppressants are related to male infertility, especially those that prevent cell proliferation, such as azathioprine, mycophenolate mofetil, and cyclophosphamide (LEROY *et al.*, 2015).

In this regard, the use of immunosuppressive therapies could also have deleterious effects on fish reproduction, also impairing their application in surrogate broodstock technology. In order to access the effects of immunosuppressive therapies on fish reproduction, this study

aimed to evaluate the impact of a combination of tacrolimus and mycophenolate mofetil on male fertility of rainbow trout.

6.2. Materials and Methods

6.2.1. Fish rearing conditions and ethical statements

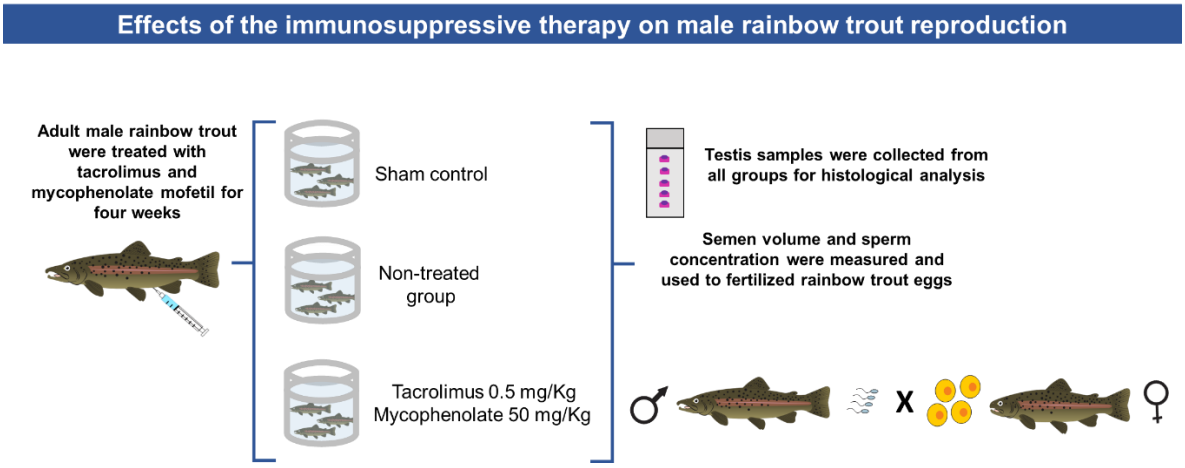
This study was conducted at Salmonid Experimental Station at Campos do Jordão, UPD-CJ (Campos do Jordão, Brazil) and the School of Veterinary Medicine and Animal Science. of University of São Paulo, FMVZ-USP. All animals used were kept in $2m^3$ round tanks with constant water flow at 13 °C (+/- 2°C) under natural photoperiod conditions. Animals were fed commercial food twice a day during the entire experiment. All procedures were carried out in accordance with the guidelines for the care and use of laboratory animals of School of Veterinary Medicine and Animal Science of University of São Paulo, certificate CEUA-6973220618.

6.2.2. Treatment with emulsion-containing tacrolimus and mycophenolate mofetil

Tacrolimus and mycophenolate mofetil were mixed in one single emulsion solution. Both immunosuppressive agents were dissolved in NaCl solution 0,7% and mixed with soil vegetal oil containing 1% of emulsifier PEG 30 Dipolyhydroxystearate (Cithrol DPHS or Arlacel P135, Croda, UK). The oil-water phase mixtures were vortexed for 30 minutes until acquiring a white-fluid solution. Tacrolimus and mycophenolate mofetil dosage used was previously established at 0.5 mg/Kg tacrolimus and 50 mg/Kg, respectively.

Twenty-four adult male rainbow trouts were selected, and their testicular content were drained. Then, they were divided in three groups of 8 animals. The first group received the immunosuppressive treatment, the second group received the emulsion solution without the immunosuppressants, and the third was used as sham control. The first and second groups received weekly intracelomatic injections of the emulsion during four consecutive weeks (**Figure 23**).

Figure 23 – Scheme of the treatment of male rainbow trout with immunosuppressive therapy



Source: YOSHINAGA, T.T, 2022

6.2.3. Reproduction parameters of male rainbow trout and progeny test

At the end of the treatment, milt samples were collected from the animals. Total milt volume and sperm concentration were analyzed, and the obtained milt samples were submitted to progeny test. Approximately 100 rainbow trout eggs were fertilized with 300ul of milt from each animal in three replicates. Spermatozoa were activated using a 0.01% NaHCO3 solution. Fertilized eggs were rinsed, hydrated in rearing water for 20 minutes, and transferred to an incubator with UV-treated water at 10°C. The fertilization, eyed-egg stage, and hatchling percentage were quantified in each replicate.

6.2.4. Conventional histology

Testicular samples from the treated animals were collected and fixed with Bouin's solution at 4 °C for 24 hours. Then, they were embedded in paraffin and sliced in 5-µm thickness sections. Testis sections were stained with hematoxylin and eosin (HE) for morphological observation of testicular tissue.

6.2.5 Statistical analysis

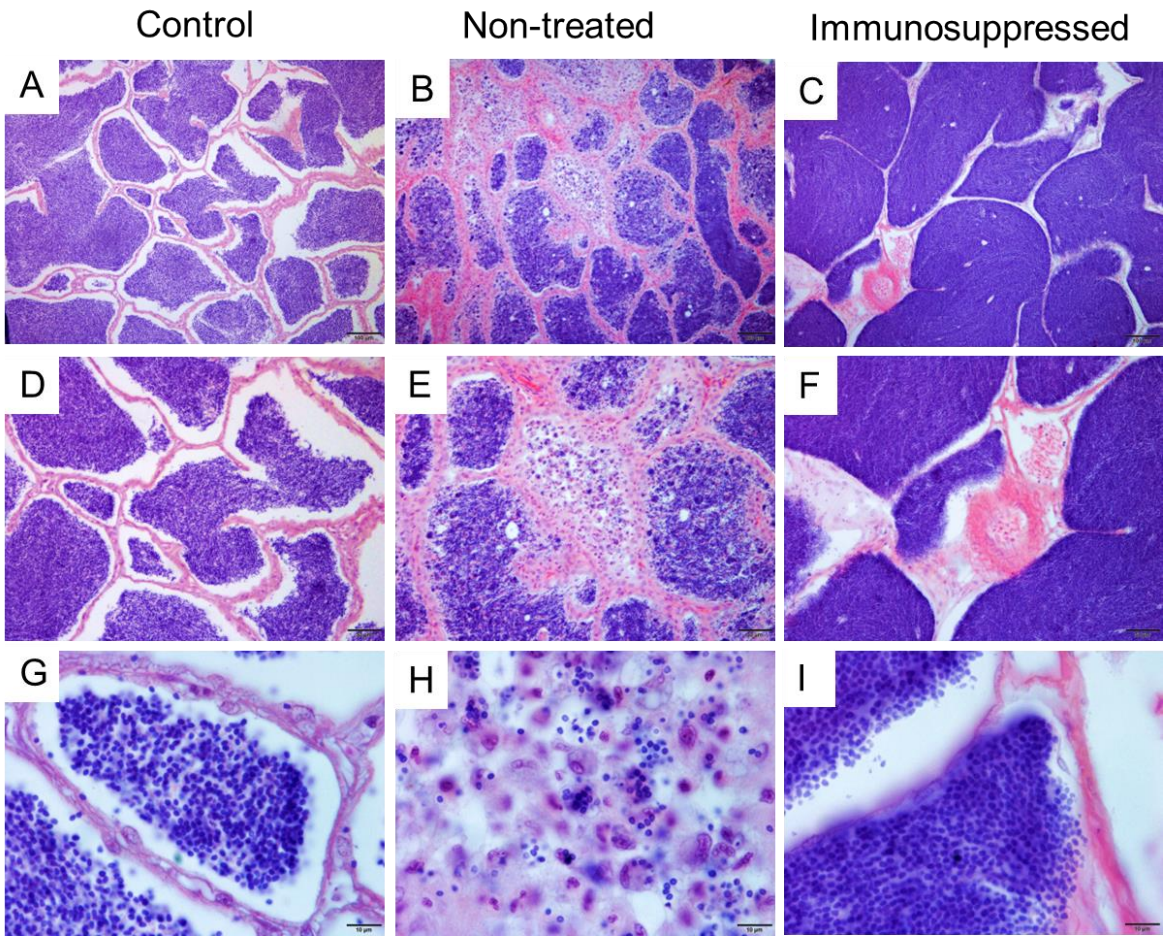
All data were analyzed in GraphPad Prism 9 (GraphPad Software, USA) using One-way ANOVA, followed by Tukey's multiple comparison test. spermatozoa parameters were compared using Student's t-test. The level of significance was considered for $p \leq 0.05$. All data were presented as mean ±S.E.M.

6.3. Results

3.1 Histological analysis from testis

Testicular tissue from control animals presented a normal morphology and higher quantity of spermatozoa similarly to the observed in the immunosuppressed treated animals (**Figure 24A**). However, testicular tissue from non-treated animals presented lower quantities of spermatozoa and signs of testicular regression of the cystic compartment. (**Figure 24B**).

Figure 24 – Histological analysis from the treated animals

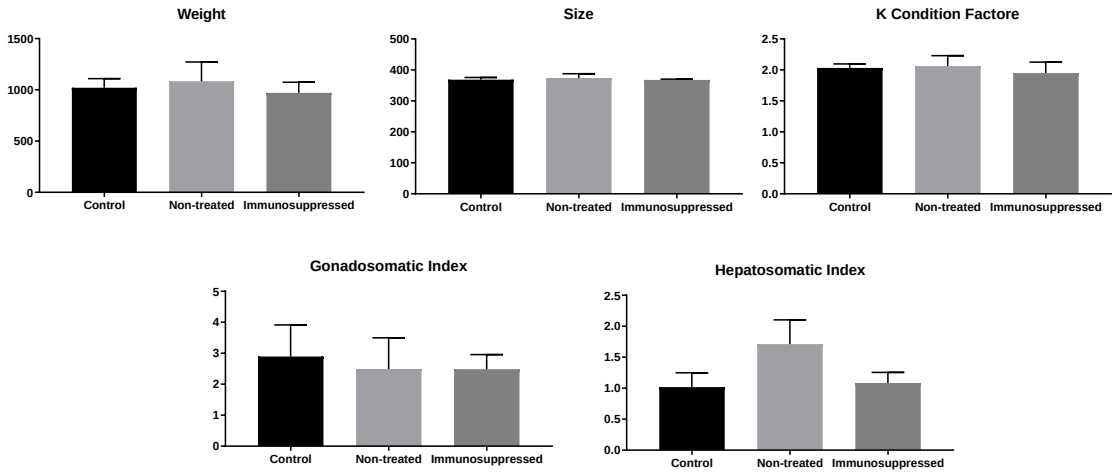


Source: YOSHINAGA, T.T, 2022

Legend: Histological analysis of the gonads from treated animals. Testicular tissue stained with hematoxylin/eosin. (A-C) panoramic overview of the testis from control, non-treated, and immunosuppressed animals. (D-I) Magnification showing testicular cyst containing spermatozoa in control and immunosuppressed group, while testicular cyst of the non-treated group presenting signs of damage cells. Scale bars from A-C indicates 100 μm, D-F 50 μm, and G-I 10 μm.

No differences were observed in growth parameters in the control group compared to both non-treated and treated group at the end of the treatment (**Figure 25**). In addition, K condition factor, gonadosomatic index, and hepatosomatic index also did not differ among the groups (**Figure 25**).

Figure 25 – Growth parameters of the treated animals



Source: YOSHINAGA, T.T, 2022

Legend: Different letters represent significantly difference for $p \leq 0.05$ One-way ANOVA analysis followed by Tukey's test. Data are presented as mean ± SEM.

6.3.1 Reproductive parameters

Before de treatment, milt volume from all animals were measured presenting similar quantities in all animals (**Table 6**). After the treatment, control animals showed higher milt volume compared to treated animals though non-treated animal did not produce milt after the treatment (**Figure 26A**). On the other hand, sperm concentration from immunosuppressed animals did not differ from control group (**Figures 26A**).

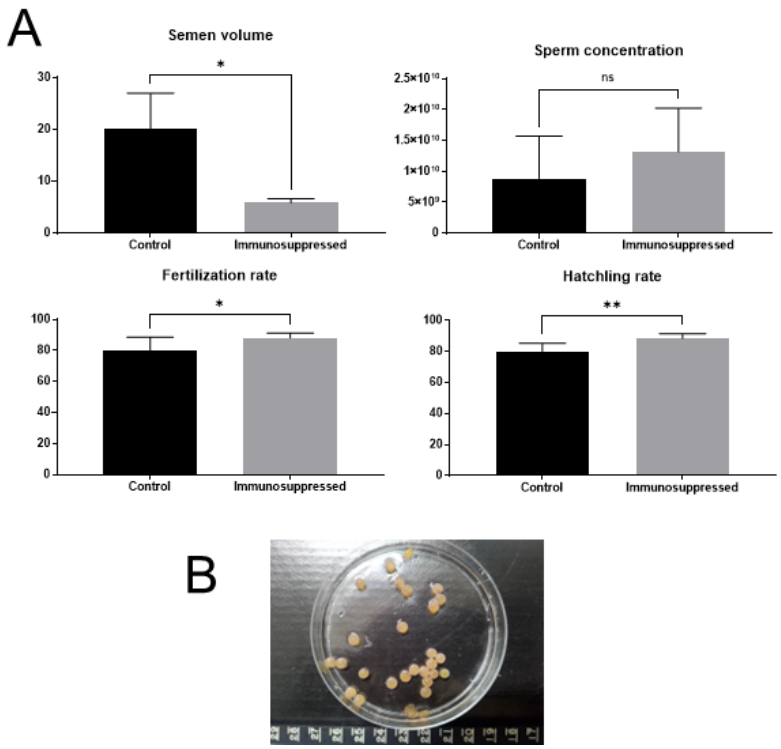
Table 6 – Data from rainbow trout males prior the treatment

Weight	Size	K condition factor	Milt volume
1062.7 ±119.7g	372±23mm	2.0±0,25	12.4±6 ml

6.3.2 Progeny test

Progeny tests using the milt from treated animals demonstrated higher fertilization and hatchling rates compared to control group. No abnormalities were observed in the progeny derived from immunosuppressed males (Figure 26B).

Figure 26 - Milt volume, sperm concentration, fertilization, and hatchling rates



Source: YOSHINAGA, T.T, 2022

Legend: (A) Milt volume, sperm concentration, fertilization, and hatchling rates from rainbow trout males treated with the immunosuppressive therapy for four-weeks. Different letters represent significantly difference for $p \leq 0.05$ Student's T tests. Data are presented as mean ± SEM.

6.4. Discussion

The action of immunosuppressive agents in fish has demonstrated similar effects as those found in mammals (XING *et al.*, 2017; YOSHINAGA *et al.*, 2021). Nevertheless, there are concerns related to the side effects of these compounds, such as the susceptibility to diseases and reproductive impairment, which is not desirable on germ cell transplantation experiments. In this study, we evaluated the effects of an emulsion containing tacrolimus and mycophenolate mofetil on rainbow trout male fertility. Treated animals presented lower milt volume but better progeny tests compared to control group. These results suggested that treatment with those

immunosuppressive drugs may not affect gametogenesis and probably may be used for donor-derived gametes production in germ cell transplantation or subcutaneous testicular grafts.

Interestingly the non-treated group was not able to produce gametes at the end of treatment. Differently from other salmonids, rainbow trout does not die after the spawning, and after a rest period the animals can be spawned again (BUTZGE *et al.*, 2021). Although, external factors such as handling or temperature stress could influence male reproductive performance, in this case the emulsion adverse effects directly affected the fish reproduction physiology. Emulsion solutions have been used to deliver vaccines due to their effects on immune system (O'HAGAN; TSAI; BRITO, 2013). The histological analysis from this group demonstrated signs of testicular regression, a process that could be accelerated by emulsion solutions in non-treated animals.

Treated animals that received emulsion solution containing tacrolimus and mycophenolate mofetil, were able to spawn at the end of the treatment. Although they presented lower milt volume, the sperm concentration did not differ from the control group. Moreover, the progeny tests conducted using the milt produced by treated animals were showed better fertilization and hatchling rates compared to control group, which suggests that immunosuppressants may not have severe effects on spermatogenesis as reported by other studies in mammals (DROBNIS; NANGIA, 2017). Nevertheless, an intriguing question was the fact of the immunosuppressed group produced functional spermatozoa in contrast to non-treated group. Although, the emulsion solution may affect spermatogenesis in non-treated animals, these results may rely on the interaction of the immune system in the gonads, such as the influence of the reproductive hormones.

The interaction between immune and reproductive system in fish is not well understood and this association has not been explored so far, especially in the immune mechanism of testicular regression. The spermatogenesis itself produces highly immunogenic spermatozoa that can be attacked by autoimmune mechanisms (JACOBO *et al.*, 2011; STEIN-STREILEIN; CASPI, 2014), but this is overcomed by immune privilege properties of the testis that avoid damage to the gametes (KAUR; MITAL; DUFOUR, 2013; KAUR; THOMPSON; DUFOUR, 2014). Differently from mammals, spermatogenesis in fish occurs in a cystic form and they lack supportive organs such as epididymis and other structures present in the mammalian reproductive tract that helps to isolate spermatozoa from the immune system (SCHULZ *et al.*, 2010). Thus, fish spermatogenesis requires other forms to avoid the spermatozoa to be attacked by the immune system. Although, sex steroid hormones were demonstrated to decrease immune

system function in fish (BUPP; JORGENSEN, 2018), this general immunosuppression may be necessary to allow spermatogenesis to occur during the spawning season.

In conclusion, immunosuppressive treatment by tacrolimus and mycophenolate mofetil did not affect spermatogenesis or fertility in rainbow trout after four weeks treatment. Although these are preliminary results, the immunosuppressive treatment may not hamper the success of germ cell transplantation studies or testicular grafts experiments.

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7. Final considerations

In this study we demonstrated the use of immunosuppressive treatment in xenogeneic germ cell transplantation and allogeneic subcutaneous testis graft in rainbow trout, as well as the effect of immunosuppressive therapy on male reproduction. Direct feed supplementation with tacrolimus may not be the best way to achieve immunosuppressive effects in fish larvae, and a different method may be more efficient to deliver these substances in fish at this stage of development. On the other hand, the injection of immunosuppressants using emulsion solution in the adult animals seems to be effective. Tacrolimus and mycophenolate mofetil decreased gene expression of immune-related markers and the acute inflammation of testicular allografts. Nevertheless, the maintenance of the grafted testis may require other factors acting together with the immunosuppression itself. Our results demonstrate that both reproductive stimuli and the immunosuppression of the host are essential to the testicular graft development as well as for germ cell transplantation.

The use of immunosuppressive agents in fish raises concerns about the susceptibility to diseases and infections and their side effects on reproduction. Although side effects in embryos and larvae are more prominent, their use in adult animals appeared to be safe. Regarding to its effects on fish reproduction, the immunosuppressive therapy of tacrolimus and mycophenolate did not inhibit or impaired spermatogenesis and the male fertility of treated animals. Thus, the immunosuppression may be a promising way to improve reproductive biotechnologies in fish, especially in testicular allo and xenografts. Combined use of a full mature animal with immunosuppression may allow the graft testis maintenance. Another consideration is that the graft testis develops according to the sexual maturation of the grafted animal and produces only donor-derived gametes.

Finally, the immunosuppression of the host may be the key to improve reproductive biotechnologies in farther-related species. Although the use of immunosuppressive agents in fish remains incipient, their application for surrogate broodstock technology is promising, especially for the reproduction of endangered fish species.

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