Neotropical Ichthyology

Original article

https://doi.org/10.1590/1982-0224-2025-0213

Cyclosporine A administration in chemically sterilized triploid *Astyanax altiparanae* to prevent cell rejection for future interspecific transplantation of fish germ cells

Giselle P. Pessoa[1,2], Lucia S. López[1,2], Amanda P. S. Silva[2], Silvio C. A. Santos[3], George S. Yasui[2,4], José A. Senhorini[1,2,†] and Paulo S. Monzani[1,2]

Correspondence:
Giselle P. Pessoa
giselle.pessoa@usp.br

Cyclosporine A (CsA) could prevent the rejection of transplanted spermatogonial cells. *Astyanax altiparanae* leukocytes were isolated and cultured at CsA concentrations of 0.10, 0.15, and 0.2 µg/µL, and viability and concentration were assessed. Juvenile triploids of *A. altiparanae* were sterilized with busulfan and administered a single dose of 20 or 40 mg/kg CsA. Control groups consisted of sterilized fish injected with the emulsion vehicle and non-injected sterilized and non-sterilized fish. Histological and molecular analyses of the head kidney were performed on days 5 and 10 after CsA administration. Gonadal histologic analysis and blood smears were assessed. Differences in leukocyte viability and concentrations were observed between treatments at 24 and 48 h of cultivation, with higher leukocyte viability at lower CsA concentrations. CsA decreased relative *interleukin-1*β expression in the head kidney, and higher CsA concentrations reduced the expression for longer. CsA did not alter the cellular composition of the head kidney and gonads. Although hemolytic anemia was observed, blood smears showed no significant reduction in leukocyte proportion. Chromosomal manipulation associated with chemical sterilization completely depleted germ cells. These preliminary results provide important information for evaluating immunosuppressants in preventing germ cell rejection in transplantation using chemically sterilized triploid recipients.

Keywords: Busulfan, Chromosome manipulation, Fish conservation, Germline chimera, Immunosuppressant.

Submitted December 17, 2025
Accepted September 22, 2026
Epub ???, 2026

Associate Editor Claudia Martinez
Section Editor Bernardo Baldisserotto
Editor-in-chief José Birindelli

1 Instituto de Biociências da Universidade Estadual Paulista, Rua Prof. Dr. Antônio Celso Wagner Zanin, 250, Distrito de Rubião Júnior, 18618-689, Botucatu, SP, Brazil. (GPP) giselle.pessoa@usp.br (corresponding author), (LSL) lsuarez@cicese.mx, (PSM) monzani.paulo@gmail.com.

2 Laboratório de Biotecnologia de Peixes, Instituto Chico Mendes de Conservação da Biodiversidade / Centro Nacional de Pesquisa e Conservação da Biodiversidade Aquática Continental, Rodovia SP-201 (Pref. Euberto Nemésio Pereira de Godoy), km 6,5, Caixa Postal 64, 13641-001, Pirassununga, SP, Brazil. (APSS) silva.amandabio@yahoo.com.br.

3 AES Tietê, Rodovia BR-153, km 139, Zona Rural, 16370-000, Promissão, SP, Brazil. (SCAS) silvio.santos@aes.com.

4 Departamento de Reprodução Animal, Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, São Paulo, SP, Brazil. (GSY) yasui@usp.br.

† *In Memoriam*

Online version ISSN 1982-0224
Print version ISSN 1679-6225

Neotrop. Ichthyol.
vol. 24, no. 4, 2026

Official Journal of the Sociedade Brasileira de Ictiologia

A ciclosporina A (CsA) pode prevenir a rejeição de espermatogônias transplantadas. Leucócitos de *Astyanax altiparanae* foram cultivados em concentrações de 0,10, 0,15 e 0,2 µg/µL de CsA, e a viabilidade e a concentração foram avaliadas. Juvenis triploides de *A. altiparanae* foram esterilizados com bussulfano e submetidos às doses de 20 e 40 mg/kg de CsA. Os controles foram peixes esterilizados injetados com emulsão e peixes esterilizados e não esterilizados sem injeção. Análises histológicas e moleculares do rim cefálico foram realizadas nos dias 5 e 10 após a administração de CsA. Histologia das gônadas e esfregaços sanguíneos foram avaliados. Houve diferenças na viabilidade e na concentração de leucócitos entre os tratamentos após 24 e 48 horas de cultivo, com maior viabilidade em concentrações mais baixas de CsA. A CsA diminuiu a expressão relativa da *interleucina-1*β no rim cefálico, e uma concentração mais elevada reduziu a expressão por um período mais prolongado. A CsA não alterou a composição celular do rim cefálico e das gônadas. Embora tenha sido observada anemia hemolítica, não houve redução significativa na proporção de leucócitos nos esfregaços. A manipulação cromossômica associada à esterilização química resultou na depleção completa das células germinativas. Esses resultados fornecem informações importantes para avaliar imunossupressores na prevenção da rejeição em transplantes de células germinativas em receptores triploides esterilizados quimicamente.

Palavras-chave: Bussulfano, Conservação de peixes, Imunossupressão, Manipulação cromossômica, Quimera germinativa.

INTRODUCTION

Germ cell transplantation biotechnology consists of isolating and transferring undifferentiated germ cells from a donor into a recipient of the same species (intraspecific transplantation) or a different species (interspecific transplantation), where they proliferate and differentiate into donor-derived gametes (Majhi *et al*., 2009; Nóbrega *et al*., 2010). Different recipient developmental stages are suitable for germ cell transplantation. In fish, germ cells can be injected into the marginal region of the blastodisc of blastula-stage embryos (Saito *et al*., 2008), into the peritoneal cavity of newly hatched larvae (Takeuchi *et al*., 2003; Okutsu *et al*., 2006), or into the gonad of sexually mature sterile recipients (Majhi *et al*., 2009; Lacerda *et al*., 2010; López *et al*., 2024).

Since the first trial in mice (Brinster, Avarbock, 1994) and subsequent adaptations in fish (Takeuchi *et al*., 2003; Okutsu *et al*., 2006), germ cell transplantation has promising applications in fish reproductive biotechnology for producing commercially valuable fish species. Particularly, for slow-maturing species, germ cell transplantation allows the use of faster-maturing surrogates as recipients, accelerating the production of donor-derived gametes (Nagasawa *et al*., 2019; Franěk *et al*., 2022), making it a potential technology for the maintenance and propagation of the competent germline of endangered species (Li *et al*., 2016).

Germ cell transplantation in adult fish is a relatively simple technology (Majhi *et al*., 2009; Lacerda *et al*., 2010). However, it requires the generation of sterile recipient fish, free of endogenous germ cells in their gonads, allowing the transplanted cells to colonize the depleted recipient gonad effectively and to prevent simultaneous production of donor- and recipient-derived gametes in non-sterile recipients, which could subsequently result in hybrid progeny (Brinster *et al*., 2003; Majhi *et al*., 2009; Ryu *et al*., 2022). Physiological and immunological compatibility with the donor, rapid growth, short reproductive cycle, and easy management in captivity are also key biological features sought in recipients to ensure transplantation effectiveness (Majhi *et al*., 2009).

Several methods can produce fish recipients, including hybrid generation through interspecific crossing and triploids via chromosome manipulation (Adamov *et al*., 2017; López *et al*., 2026). Chemical sterilization using antineoplastic drugs at high temperatures can also produce sterile recipients (López *et al*., 2026). Busulfan is the most used drug for this purpose, as it inhibits the proliferation and differentiation of spermatogonial stem cells and promotes apoptosis of a series of spermatogenic cells (Chen *et al*., 2018). However, this method may not achieve long-term sterilization, as the recipient germ cells may proliferate again after a few months (Zohni *et al*., 2012; de Siqueira-Silva *et al*., 2015).

Cellular rejection of cell transplants is an immunological response that represents a significant obstacle to cell transplantation efficiency (Yoshinaga *et al*., 2021). When transplantation is performed into immunologically immature recipients during the blastula and newly hatched larval phases, the undifferentiated germ cells transplanted do not undergo cellular rejection due to possible endogenous tolerance, leading to a higher success rate in gamete production (de Siqueira-Silva *et al*., 2015). In adult recipients, the immune response to germ cells after a few weeks post-transplantation reduces the percentage of recipients able to generate gametes from the donor (Yoshinaga *et al*., 2021). The immunosuppressant CsA is a cyclic polypeptide of fungal origin, and it can bypass the immunological barrier, increasing transplantation success. However, CsA is an aggressive drug, mainly due to its nephrotoxic effects, despite other adverse effects that have been reported (Demirci *et al*., 2024).

This study aimed to determine whether different CsA concentrations could effectively suppress the immune response in the neotropical fish *Astyanax altiparanae* while minimizing adverse effects. To test this hypothesis, we used *in vitro* leukocyte assays to identify immunosuppressive CsA concentrations, which we then evaluated in vivo in chemically sterilized triploid recipients.

MATERIAL AND METHODS

Material. Males and females of *A. altiparanae* were collected in fish farming tanks filled with water from local hydrological sources from a tributary of the Mogi Guaçu River, at the National Center for Research and Conservation of Continental Aquatic Biodiversity of the Chico Mendes Institute for Biodiversity Conservation (CEPTA/ICMBio), in Pirassununga, São Paulo State, Brazil.

Chromosome manipulation. Breeders were selected based on secondary sexual characteristics, and females, with a bulging abdomen and red papilla, and males, with a narrow abdomen and spike in the anal fin, were subjected to hormonal induction using crude carp pituitary extract in a 0.9% NaCl solution. Females received an initial dose of 0.5 mg/kg, followed by a second dose of 5 mg/kg six hours later. Males received a single dose of 2 mg/kg, given simultaneously with the second dose administered to females. Fish were kept in a 40 L aquarium filled with dechlorinated water from the domestic water supply, using a closed recirculation system with continuous aeration, maintained at 28°C during hormonal administration.

Six hours after hormonal induction, the oocytes were manually collected, and the extracted semen was added to modified Ringer's immobilizing solution (128.3 mM NaCl, 2.6 mM KCl, 1.8 mM $CaCl_2$, 2.1 mM $MgCl_2$, and 2.4 mM $NaHCO_3$). Semen was added to the oocytes, and fertilization was performed by adding aquarium water while rotating the plate in circular motions for 1 min. The fertilized embryos were put in plastic containers lined with a 100 µm nylon mesh at the bottom and kept in a water bath at 26°C for up to 2 min after fertilization.

For triploidization, embryos were immersed in a 40°C water bath for exactly 2 min, according to the optimized protocol described by Adamov *et al.* (2017). After the heat shock, eggs were immediately returned to the 26°C water container.

The triploid larvae were kept in a 40 L aquarium at 28°C under continuous aeration and were fed daily with *Artemia salina*. They were subsequently transferred to masonry tanks and given commercial feed for two months. Analysis of triploidization was performed as previously described (Xavier *et al*., 2017) using flow cytometry (CyFlow Ploidy Analyzer, Partec, GmbH, Germany).

Chemical sterilization of juvenile triploid *A. altiparanae*. The experimental procedures for chemical sterilization are schematized in Fig. 1. Two groups of 10 male triploids of *A. altiparanae* were acclimatized in aquariums containing 40 L of dechlorinated water, with constant aeration at 28°C for 1 week. The water temperature was raised by 1°C each day until it reached 35°C, and the animals were kept at this temperature for 1 week. Two male gonads were used as controls before the temperature rise for the histological analysis to examine their overall cell composition. The chemical sterilization was achieved through three administrations of 50 mg/kg busulfan, injected into the intracoelomic cavity of the recipients at seven-day intervals. Ten days after the last application, the temperature was reduced by 1°C each day until it reached 28°C. The fish received commercial feed once daily throughout the experiment.

Gonad samples were collected from fish and fixed in Bouin's solution (Cinetica, Laboratórios Adria, Brasil) for histological analysis. Histological processing for inclusion in paraplast involved dehydration in a graded ethanol series, followed by immersion in xylene. The samples were embedded in paraplast. Once hardened, the samples were sectioned at 5 µm using a microtome (Leica Biosystems #RM2235, Heidelberger, Germany). Paraffin sections were dewaxed by immersion in xylene, rehydrated in a decreasing alcohol series, and stained with hematoxylin and eosin. The histological sections were observed under an optical microscope (Nikon Eclipse Ni, Tokyo, Japan).

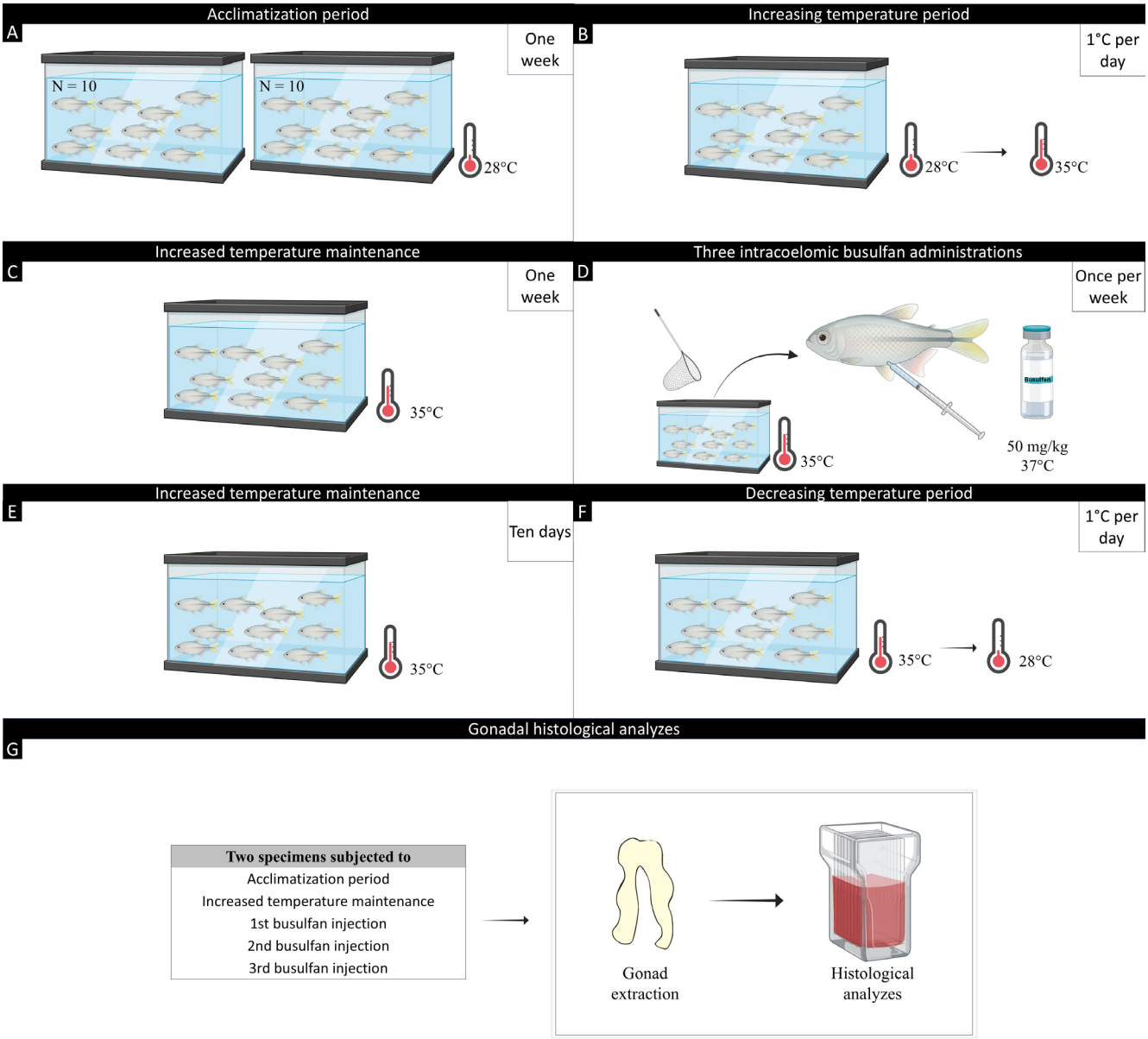

FIGURE 1 | Experimental design illustration of the chemical sterilization protocol for juvenile triploid *Astyanax altiparanae*. (**A**) Acclimatization period of male triploids (N = 20) at 28°C for 1 week. (**B**) Gradual temperature increase (+1°C/day) to 35°C. (**C**) Maintenance at 35°C for 1 week. (**D**) Busulfan administration (50 mg/kg, once weekly for 3 weeks). (**E**) Temperature maintenance at 35°C for ten days. (**F**) Gradual temperature decrease (-1°C/day) to 28°C. (**G**) Gonadal histology at 5 time points (N = 2 per time point).

Isolation and culture of *A. altiparanae* leukocytes. We collected approximately 2 mL of peripheral blood and placed it in a tube with 30 µL of 0.5 M EDTA at 4°C. The blood was diluted 1:1 in RPMI 1640 with L-glutamine and enriched with 10% fetal bovine serum. 4 mL of the blood solution was deposited into 4 mL of separation medium density Histopaque©1077, to isolate leukocytes. The sample was centrifuged (Centrifuge 5702 – Eppendorf, Hamburg, Germany) at 700 x g for 30 min, and the

leukocyte button (Fig. **S1**) was recovered and transferred to a new tube. Then, 1 mL of enriched RPMI medium was added and the sample was centrifuged at 300 x g for 5 min. This procedure was repeated twice. The supernatant was discarded, and 500 µL of enriched RPMI was added to suspend the leukocyte material. 10 µL of material was collected and analyzed for cell concentration and viability in a Neubauer blood cell chamber under a microscope (Nikon-Eclipse Ni). Leukocytes were visualized (Nikon-Eclipse Ni, Tokyo, Japan) and photographed (Nikon DSRi2, Nikon, Tokyo, Japan). We adjusted the cell concentration to $4.5x10^5$ cells/mL.

Leukocytes were cultured in triplicate in a 48-well plate (Greiner Bio-One Brasil) at concentrations of 0.10 µg/µL, 0.15 µg/µL, and 0.2 µg/µL CsA, and without CsA as a control. A stock solution of CsA at 2 mg/mL was prepared by diluting it first in ethanol and then in RPMI. For each series of wells, 100 µL of cell solution was deposited in 100 µL of CsA solution at different concentrations to obtain final concentrations of 0.10 µg/µL, 0.15 µg/µL, and 0.2 µg/µL. For the control series, 100 µL of enriched RPMI was added to 100 µL of cell solution. The culture plate was maintained in a CO_2 incubator (Sanyo COM–18AIC UV CO2 Incubator, SANYO Electric Co., Ltd., Japan) at 30°C and 5% CO_2.

Leukocyte viability was analyzed 24 and 48 hours after the start of the culture. We collected 10 µL of solution from each well and stained it with 2 µL of 0.4% Trypan blue. The cells were deposited in the Neubauer chamber and counted under a microscope.

***In vivo* CsA administration in juvenile triploids of *A. altiparanae* chemically sterilized.** The effects of CsA in fish were evaluated by (i) the expression of *interleukin-1*β in head kidney samples; (ii) the quantity and integrity of the hematopoietic cells from the head kidney; (iii) the production of germ cells by the gonads; and (iv) the proportion of leukocytes in smear blood samples. The Fig. 2 presents the experimental design.

Gonads and head kidneys of fish not treated and treated with busulfan, after five days of acclimatization to 28°C, were collected and used as control samples, and histology was assessed as described in "*Chemical sterilization of juvenile triploids A. altiparanae*". Parts of the head kidney were frozen at -80°C for subsequent RNA extraction.

Three groups of eight chemically sterilized fish were treated with CsA: a control group (emulsion without CsA) and groups treated with 20 mg/kg and 40 mg/kg CsA. Each group maintained two surplus animals to ensure adequate sampling, considering the nephrotoxicity and lethality of CsA.

The emulsion solution was prepared by combining equal volumes of saline solution and absolute ethanol, then homogenizing the mixture with fish oil at a 1:1 ratio in a vortex for ten minutes. CsA solutions at 20 and 40 mg/kg were prepared using solutions containing 2 and 4 mg CsA/mL, which were then homogenized in an emulsion solution to produce the injectable immunosuppressant. The administration was performed into the coelomic cavity of fish from the control and CsA-treated groups, and maintained and fed in 40 L aquariums at 28°C with constant aeration. We collected three fish from each group on the 5th and 10th days after injection of CsA and emulsion. We collected gonads, blood, and head kidney from each fish.

We prepared a blood smear from each sampled animal, fixed the slides in absolute ethanol for 5 min, and stained them in Giemsa solution for 30 min, using 2 drops of dye per mL of distilled water. One hundred leukocytes were counted directly using

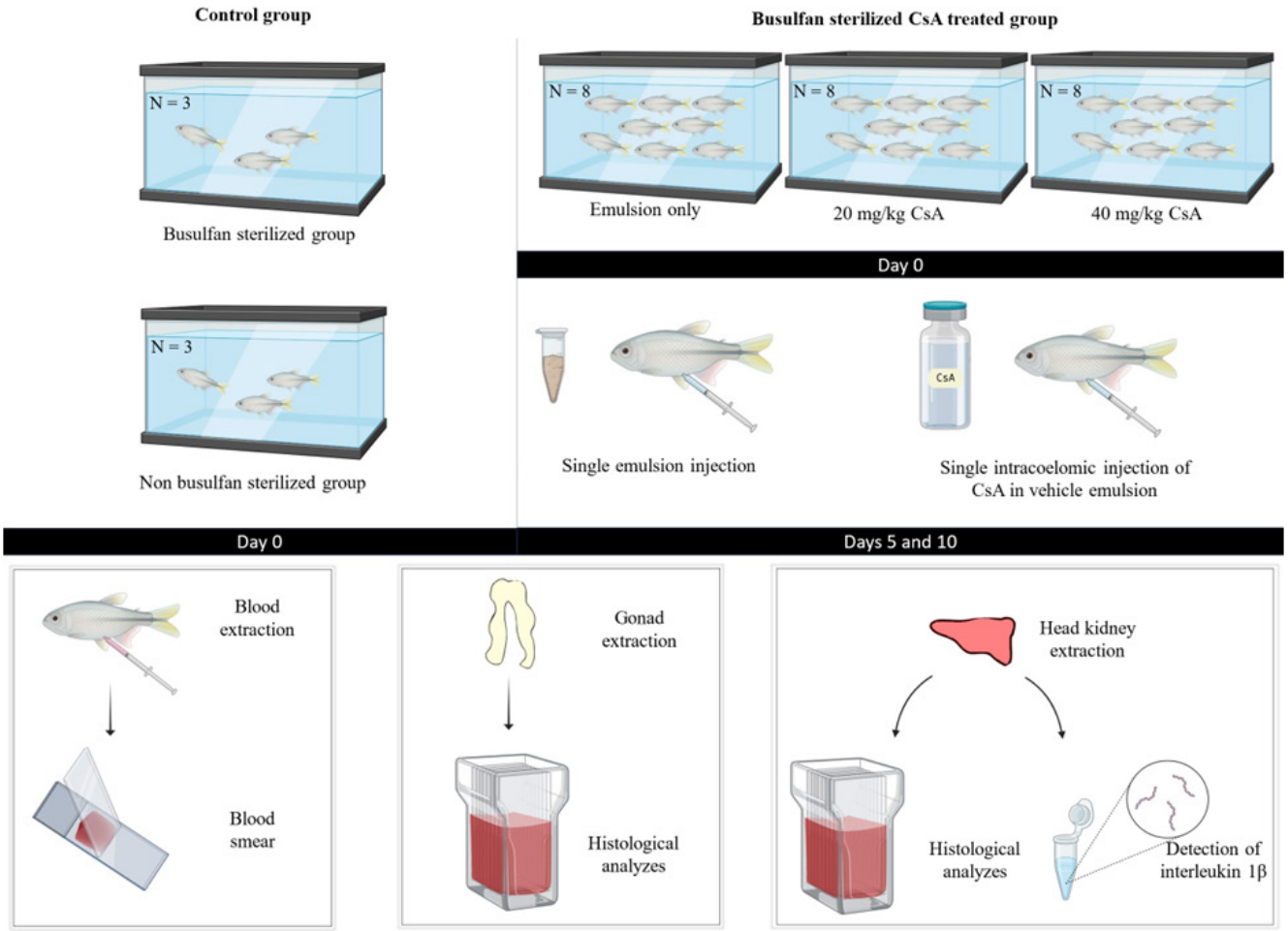

FIGURE 2 | Illustration of *in vivo* CsA administration experiment in chemically sterilized juvenile triploids of *Astyanax altiparanae*.

microscopy in the second third of the smear (Ranzani-Paiva, Godinho, 1985). They were visually segregated based on nuclear and cytoplasmic features, following the classification previously described (Ranzani-Paiva, Godinho, 1985; Sarrafzadeh *et al.*, 2014). The percentages of neutrophils, eosinophils, basophils, lymphocytes, and monocytes from smears from each treatment were noted.

The gonads and head kidney fractions were subjected to histological processing, as described in "*Chemical sterilization of juvenile triploids A. altiparanae*". The lymphohematopoietic tissue of the head kidney was morphometrically analyzed using ImageJ software. Images were automatically linearized and converted to grayscale, and cells were counted manually. We investigated whether cell density and hematopoietic cell proliferation varied among the different CsA treatments in the head kidney. Furthermore, histological sections of proximal and distal tubules of the renal stroma were also assessed.

The expression of the *interleukin-1*β gene was evaluated using both conventional and real-time PCR. Total RNA samples from each collection were pooled to create a representative sample. Each pool contained the same RNA concentration from each sampled individual. The samples were macerated using a pestle and homogenized by pipetting. RNA was extracted with TRIzol reagent, treated with DNase I, quantified using a UV/VIS microfluidic spectrophotometer QIAxpert (Qiagen, Hilden, Germany), and stored at -80°C. The first strand of cDNA synthesis was performed using the High-Capacity cDNA Reverse Transcription Kit, following the manufacturer's instructions.

PCR reactions were prepared using *interleukin-1*β primers and the endogenous *18S rRNA* (conventional PCR) or β-*actin* (qPCR) gene of *A. altiparanae* (Tab. 1). The primer sequences for *18S rRNA* and β-*actin* were designed in our laboratory, and the *interleukin-1*β primer sequences were previously published (Levy-Pereira *et al.*, 2021). The PCR reaction was performed in the thermocycler (Rotor-Gene Q, Qiagen) with the following cycling parameters: 94°C for 5 min, 35 cycles of 94°C for 45 s for denaturation, 55°C for 30 s for annealing, 72°C for 30 s for extension, and 72°C for 10 min. Electrophoresis was carried out in 0.5X TBE solution at a constant voltage of 70V on a 2% agarose gel. The amplification products were visualized using a UV transilluminator (Accurais E4000 Smartblue, Benchmark Scientific) and photographed.

The relative quantitative expression analysis of *interleukin-1*β was performed using the Applied Biosystems 7500 Fast Real-Time PCR thermal cycler System (Applied Biosystems, Thermo Fisher Scientific Inc., Foster City, California, United States), using the standard curve method for relative quantification. We normalized quantification to the endogenous β-*actin* control and expressed relative quantification using the control group treated with emulsion as the calibrator. Standard curves for β-*actin* and *interleukin-1*β were constructed by serial dilution at 1:3 using cDNA at 648, 216, 72, 24, and 8 ng. The curves were created and analyzed using a negative control. All samples were amplified in triplicate. Each reaction contained 10 µL of QuantiNova SYBR Green PCR Master Mix (Qiagen, Hilden, Germany), 0.1 µL of ROX dye cycler (Qiagen, Hilden, Germany), 1 µL of the primer mix (250 nM of each), 400 ng cDNA, and nuclease-free water up to 20 µL of final reaction volume. Amplification conditions were: activation of DNA polymerase at 95°C for 2 min, followed by 40 cycles of 95°C for 10 s and 62°C for 30 s, followed by a melting curve from 60°C to 95°C. The 7500 Software v. 2.0.5 (Applied Biosystems, Thermo Fisher Scientific Inc.) was used. The amplification reaction efficiencies (E) were measured using the equation: $E = (10^{-1/slope}-1) \times 100$.

TABLE 1 | Sequences of primers used in the evaluation of gene expression by conventional and real-time PCR.

Primers	Sequences 5' → 3'	Amplicon (bp)
rRNA18S-F	GTCTGTGATGCCTTAGATGTC	107
rRNA18S-R	CGAACGGGTTTAAGCGGATT	
IL-1β-F	CCTCACAGCATGAGGAAGGTGG	105
IL-1β-R	GTGCTCGGTGAACTCTGTGGAG	
β-actin-F	CGTGCTGTCTTCCCATCCA	86
β-actin-R	TCACCACGTAGCTGTCCTTCTG	

Statistical analysis. We analyzed leukocyte viability and concentration at different CsA concentrations in vitro using one-way ANOVA followed by Tukey's post hoc test for multiple comparisons ($p < 0.05$) in PAST 4.03, as ANOVA is robust for balanced experimental designs. Conversely, cell density in histological preparations of the head kidney (*in vivo* CsA) and leukocyte proportions in peripheral blood (busulfan + CsA) were analyzed using non-parametric Kruskal-Wallis tests followed by Dunn's post hoc test, or Mann-Whitney U tests for two-group comparisons ($p < 0.05$) in JASP 0.19.3.0, due to the reduced sample sizes ($N = 2 – 3$ per group).

RESULTS

Chemical sterilization of juvenile triploid *A. altiparanae*. Histological analysis of the gonadal microenvironment in juvenile triploid *A. altiparanae* at 28°C (Fig. 3A) showed no sperm in the seminiferous ducts and spermatogonial cells with prominent, rounded nuclei that were mainly centrally located and abundant in both euchromatin and heterochromatin. After the temperature rise, we observed progressive germline degradation (Fig. 3B), with scarcer spermatogonia interspersed with germinal cysts, where spermatocytes were predominant. After the following busulfan administration (Figs. 3C, D, E), the gonads consisted solely of somatic cells from the cysts, surrounded by interstitial tissue.

After chemical sterilization, the fish were immunosuppressed. Histological analysis of testes collected on the fifth day after CsA administration at 20 and 40 mg/kg revealed structural characteristics similar to those following busulfan treatment, indicating gametogenic depletion (Fig. **S2**). On the 10th day after CsA injection, we did not collect gonads from either group for histological analysis because they were too small and fragile to handle. The gonads of chemically sterilized triploid groups (i) treated with CsA, (ii) treated with emulsion, and (iii) not injected with emulsion or CsA (control group treated with busulfan) were tiny, with less mass and often present on only one side of the intracoelomic cavity, in contrast to the non-sterilized triploid control group.

***Astyanax altiparanae* leukocyte *in vitro* culture.** The cellular concentration of leukocytes after recovery of the leukocyte button was $1.17x10^6$ cells/mL, with an average cell viability of 91.14%. In the cell viability analysis (Fig. 4A), we observed a significant statistical difference between treatments at 24 and 48 h of cultivation ($p = 0.0038$), and the repetitions were homogeneous within the two groups. Statistical differences in cell viability were observed after 24 h of cultivation. The viability rates at concentrations of 0.1 µg/µL and 0.15 µg/µL were higher compared to 0.2 µg/µL. After 48 h of culture, cell viability decreased slightly, with the lowest concentration (0.1 µg/µL) still maintaining the highest viability in this range. This indicates that 0.1 µg/µL is the safest concentration for *in vitro* use. Leukocyte concentrations decreased after 24 and 48 h of cultivation (Fig. 4B), with a more significant reduction at 24 h. The highest concentration of CsA resulted in the greatest cell death (Fig. 4A). Both lower concentrations showed similar cell viability, with 0.1 µg/µL being less harmful after 48 h of cell culture. *In vitro* results can inform *in vivo* administration. Based on the lowest concentration being the most suitable, a suggested concentration for in vivo testing is 100 mg/kg, which is higher than that used in *in vivo* tests in this work.

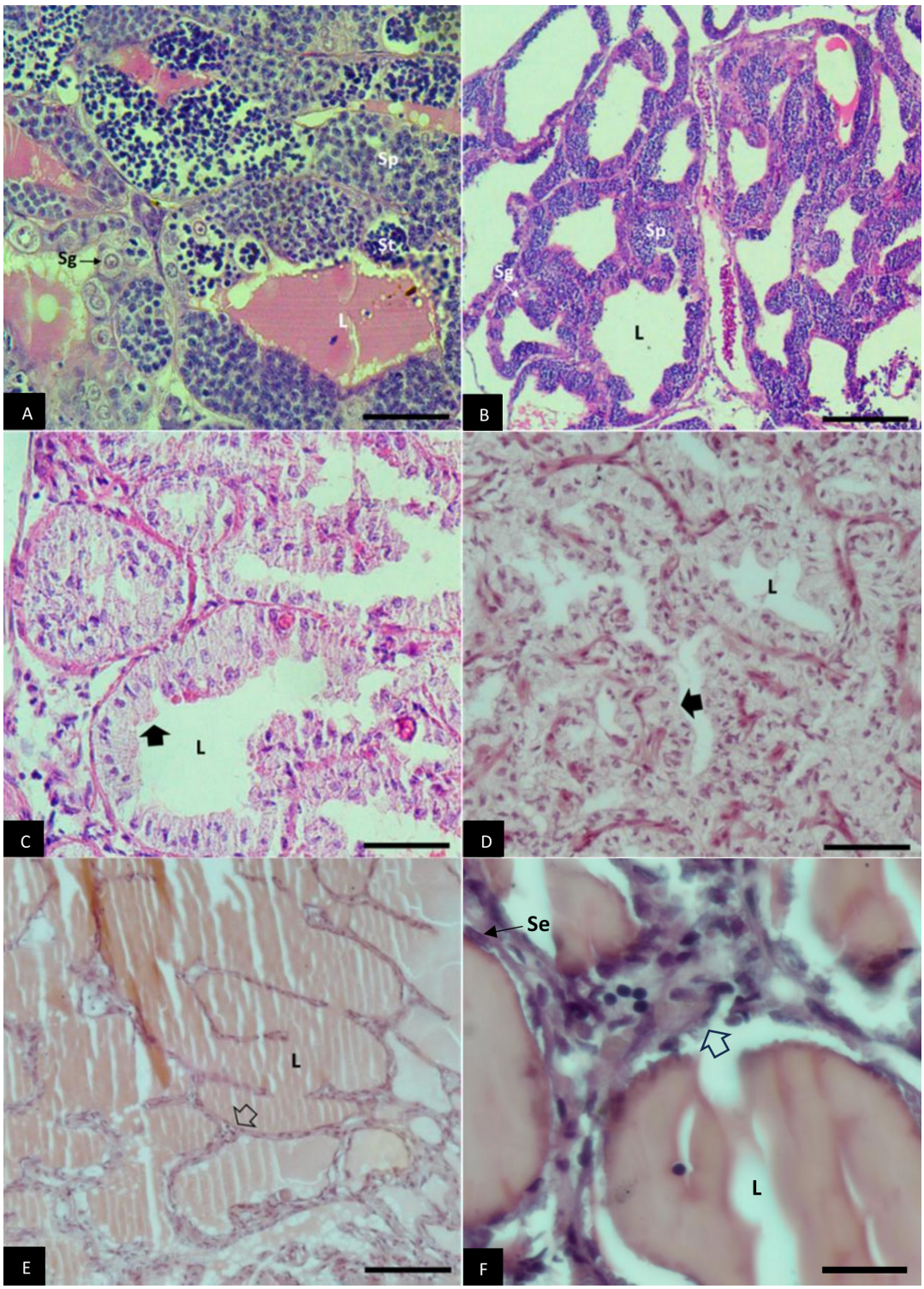

FIGURE 3 | Testicular histology of triploid *Astyanax altiparanae* chemically sterilized with busulfan at 50 mg/kg. **A**. Testicle before temperature rise, with germinal cysts containing spermatogonia (Sg), spermatocytes (Sp), spermatids (St), and lumen (L) of the seminiferous tubule without sperm. **B**. Testicle one week after increased temperature. **C**. Testicle one week after the first busulfan application, confined by the columnar epithelium (filled arrow). **D**. Testicle one week after the second busulfan application, with observed interstitial tissue (empty arrow). **E**. Testicle one week after the third application of busulfan. **F**. Detail of the testicle one week after the third application of busulfan, showing Sertoli cells (Se) around cysts with a germ-free lumen (L); empty arrow indicates interstitial tissue. **A**, **C**, and **D**: scale: 100 µm. **B** and **E**: scale: 200 µm. **F**: 50 µm.

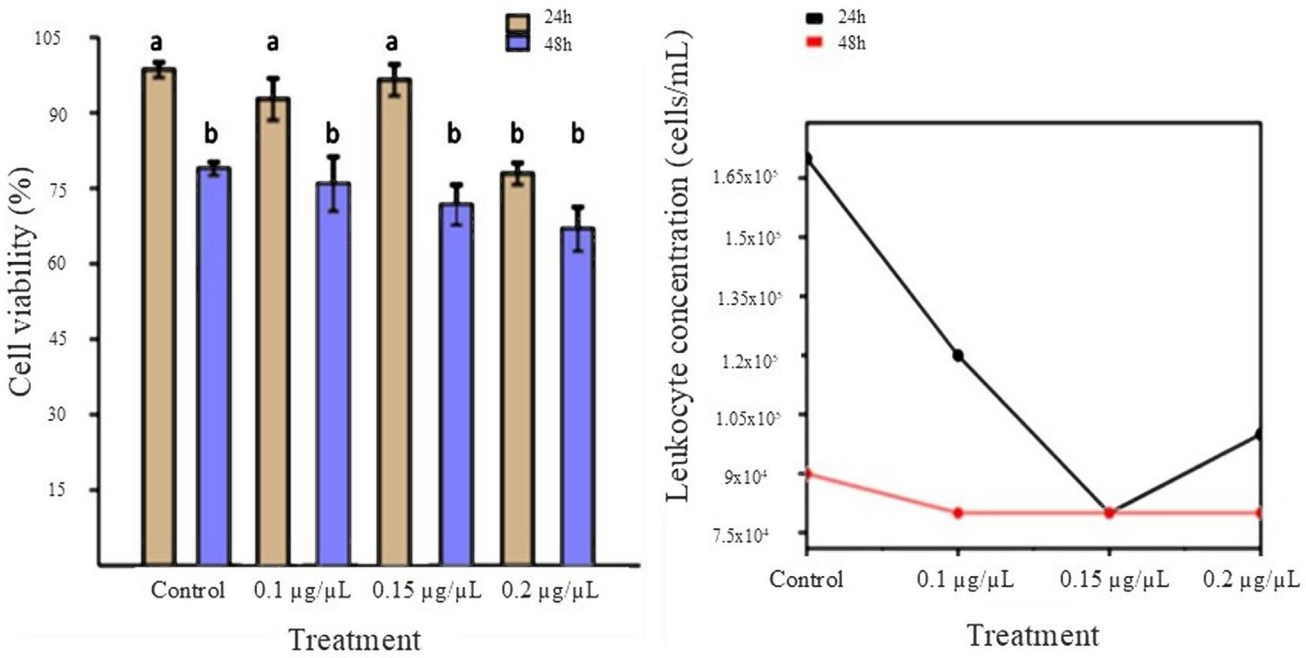

FIGURE 4 | Viabilities and leukocyte concentrations under different *in vitro* CsA concentrations. **A**. Cell viability (%) 24 h and 48 h after leukocyte cultivation under different concentrations of CsA (0.1, 0.15, 0.2 µg/µL) and the control group without CsA treatment. **B**. Leukocyte concentrations 24 and 48 h after culture *in vitro* with CsA (0.1, 0.15, 0.2 µg/µL) and the control group without CsA.

Histological analysis of *A. altiparanae* kidneys and gonads. One week after administering a single injection of CsA, the treatment groups receiving 20 mg/kg and 40 mg/kg of CsA each experienced the death of three fish. In contrast, the control group, which was only injected with the emulsion, lost one fish (Fig. **S3**). Immunosuppressant nephrotoxicity may have been the main cause of the higher lethality among the groups subjected to CsA injection. We did not collect head kidney samples from the 40 mg/kg group on day 10 for histological analysis because the organ was disrupted during sampling. We performed histological analysis of hematopoietic tissues from the head kidney (Figs. 5A, C) and the renal parenchyma, including proximal and distal convoluted tubules (Figs. 5B, D), in the control group (treated with emulsion) and CsA-treated groups. Representative images of the control groups are shown in Fig. 5 (A, C: hematopoietic tissue; B, D: renal stroma). No significant differences in cell density were observed between treatments ($p = 0.1647$), indicating that CsA did not inhibit hematopoiesis. Although the kidney cell structure remained unchanged by the immunosuppressant, the kidney solidity and integrity were compromised compared to the organs of control animals that received only emulsion or no treatment.

The gonadal histology could not be effectively evaluated. Tissue preservation in several gonadal samples was compromised, limiting the ability to obtain high-quality histological images suitable for publication. Therefore, we did not include gonadal histological analysis in the results. However, the findings indicate no apparent gonadal alterations associated with CsA administration, as shown in Fig. **S2**.

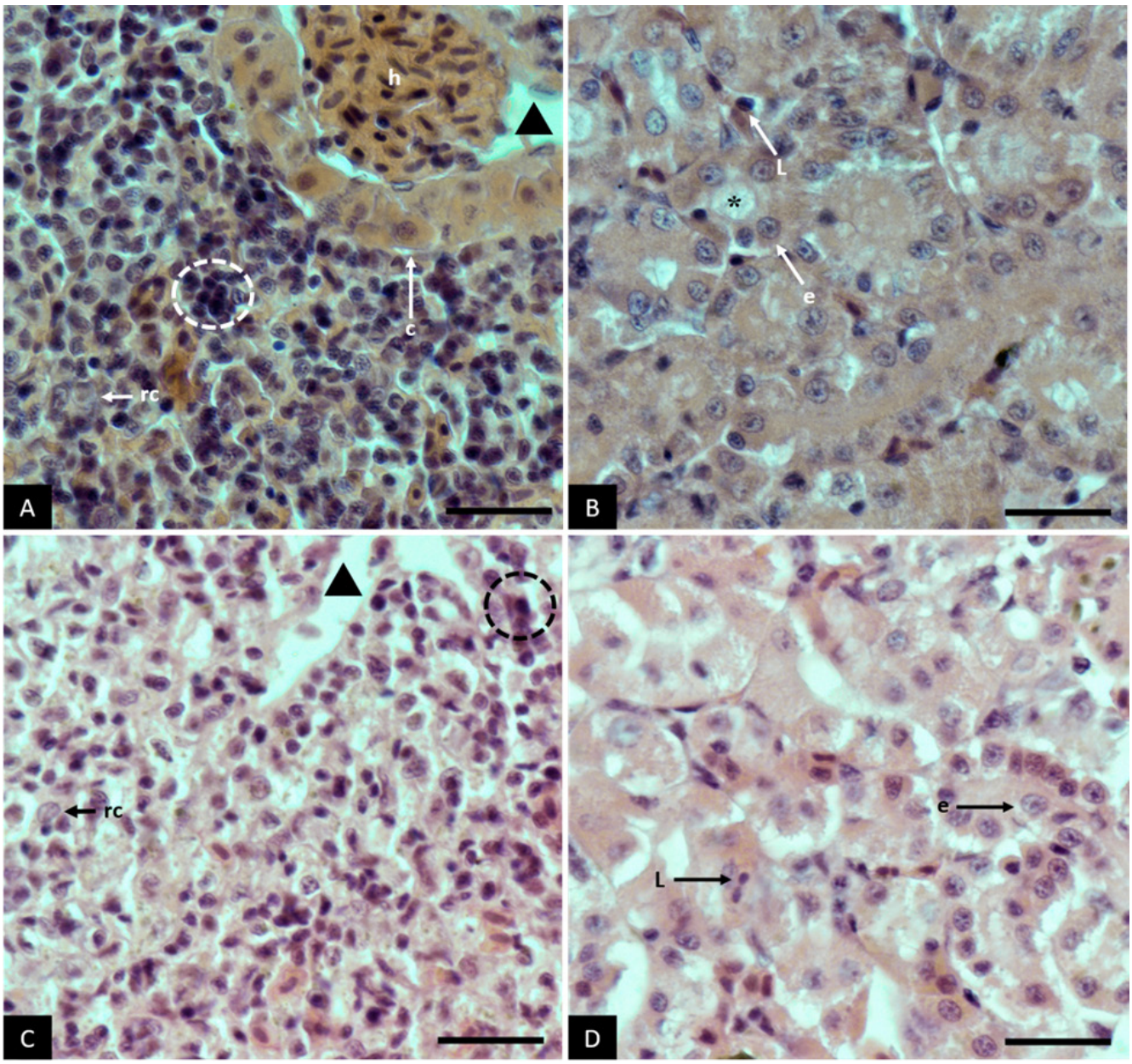

FIGURE 5 | Head kidney histology of triploids *A. altiparanae* not treated with CsA. **A, C.** Hematopoietic tissue of the control without emulsion and of the control with emulsion, respectively, showing a sinusoid (arrowhead) and a cluster of red blood cells (h), lined by cubic interrenal cells (c); melanomacrophage centers (dotted circle) and cell-matched reticular (rc) cells amid the interrenal cells of the hematopoietic tissue of the head kidney. **B, D.** Renal stroma in the head kidney of the control without emulsion and of the control with emulsion, respectively, showing convoluted proximal tubules (asterisk) with simple epithelial cells (e) and lymphocytes (L). 50 µm scale.

Blood smear. The most common leukocyte types identified were lymphocytes, with a rounded nucleus and dense chromatin, followed by neutrophils, with 3 to 5 lobes connected by a chromatin bridge. Eosinophils, distinguished by 2 lobes; basophils, with a chromatin bridge thicker than other cell types and rich in granules; and monocytes, of larger volume and a large horseshoe-shaped nucleus, were rarer. The proportions of leukocyte types in blood smears from each treatment are shown in Tab. 2 and visualized in Fig. 6. On the 10th day of treatment with 20 mg/kg of CsA, blood smears were not

taken from the surviving fish, even when collecting directly from the intracoelomic cavity after excising the head kidney, as the blood was very scarce and thin. Although blood smears were obtained, the individuals treated with 40 mg/kg CsA also exhibited these characteristics on the 10th day.

A few leukocyte cells were found in treatments of 20 mg/kg (Fig. 6D) and 40 mg/kg of CsA compared to the control groups, treated and not treated with busulfan (Figs. 6A, B), and treated with emulsion (Fig. 6C). This observation may be attributed to the blood samples, which were thinner and less abundant, possibly indicating side effects similar to those observed in humans.

Analysis of *interleukin-1β* expression in head kidneys of *A. altiparanae*. We detected amplification of the *interleukin-1*β gene in all evaluated samples (Fig. **S4**). This was also observed with the endogenous *18S rRNA* gene. Only one amplification band was observed for each evaluated gene, indicating that the primers were specific to their target sequences. For qPCR analysis, we used β-*actin* as the endogenous gene because *18S rRNA* is highly expressed.

The qPCR analysis standard curves demonstrated amplification efficiencies of 109% for *interleukin-1*β and 105% for β-*actin*, with the sample dots falling within the curve limits. The relative expression data indicate that *interleukin-1*β had the highest expression in the control group that received the emulsion treatment, measured on the 10th day after application. In contrast, the control group treated with busulfan showed approximately 20% lower relative *interleukin-1*β expression than the emulsion group. Additionally, groups treated with CsA at 20 mg/kg and 40 mg/kg showed reductions in *interleukin-1*β expression of approximately 60% and 46%, respectively, on the 5th day after immunosuppressant administration. The decrease in *interleukin-1*β expression was even more pronounced on the 10th day after application, reaching reductions of approximately 73% (20 mg/kg) and 81% (40 mg/kg) of gene expression. The relative expression plot is shown in Fig. 7.

TABLE 2 | Effect of CsA treatment on *Astyanax altiparanae* from days 0 (controls without any application), 5, and 10 (control with emulsion vehicle and CsA treatment) on the proportions of leukocyte types in the blood smear. Note: Values are expressed as mean ± SD. Statistical analysis was performed using the Kruskal-Wallis test (post hoc analysis: Dunn's test) or the Mann-Whitney *U* test for two-group comparisons. Different superscript letters indicate significant differences between groups within the same time point ($p < 0.05$). Only Basophils on Day 5 showed significant differences (Kruskal-Wallis $p = 0.039$; Dunn's test: 40 mg/kg vs. control with emulsion, $p = 0.011$).

	Control without busulfan (Day 0)	Control with busulfan (Day 0)	Control with emulsion (Day 5)	20 mg/kg CsA (Day 5)	40 mg/kg CsA (Day 5)	Control with emulsion (Day 10)	40 mg/kg CsA (Day 10)
Neutrophils [%]	21.00 ± 1.0	2.66 ± 1.52	15.00 ± 2.0	23.66 ± 2.08	20.84 ± 2.06	26.00 ±4.0	27.29 ± 2.41
Eosinophils [%]	9.00 ± 2.64	0.33 ± 0.57	13.66 ± 4.16	11.66 ± 4.04	9.86 ± 0.99	7.00 ± 2.0	4.16 ± 2.60
Basophils [%]	6.33 ± 0.57	0.66 ± 0.57	10.33 ± 2.51[a]	4.66 ± 1.52[ab]	2.74 ± 0.48[b]	6.33 ± 0.57	9.48 ± 3.55
Lymphocytes [%]	46.33 ± 2.51	95.00 ± 2.0	57.33 ± 1.52	55.66 ± 2.51	65.77 ± 3.54	56.33 ± 5.50	51.4 ± 6.22
Monocytes [%]	17.33 ± 1.52	1.33 ± 0.57	3.66 ± 1.52	4.33 ± 2.08	0.78 ± 0.67	4.33 ± 0.57	7.66 ± 2.34

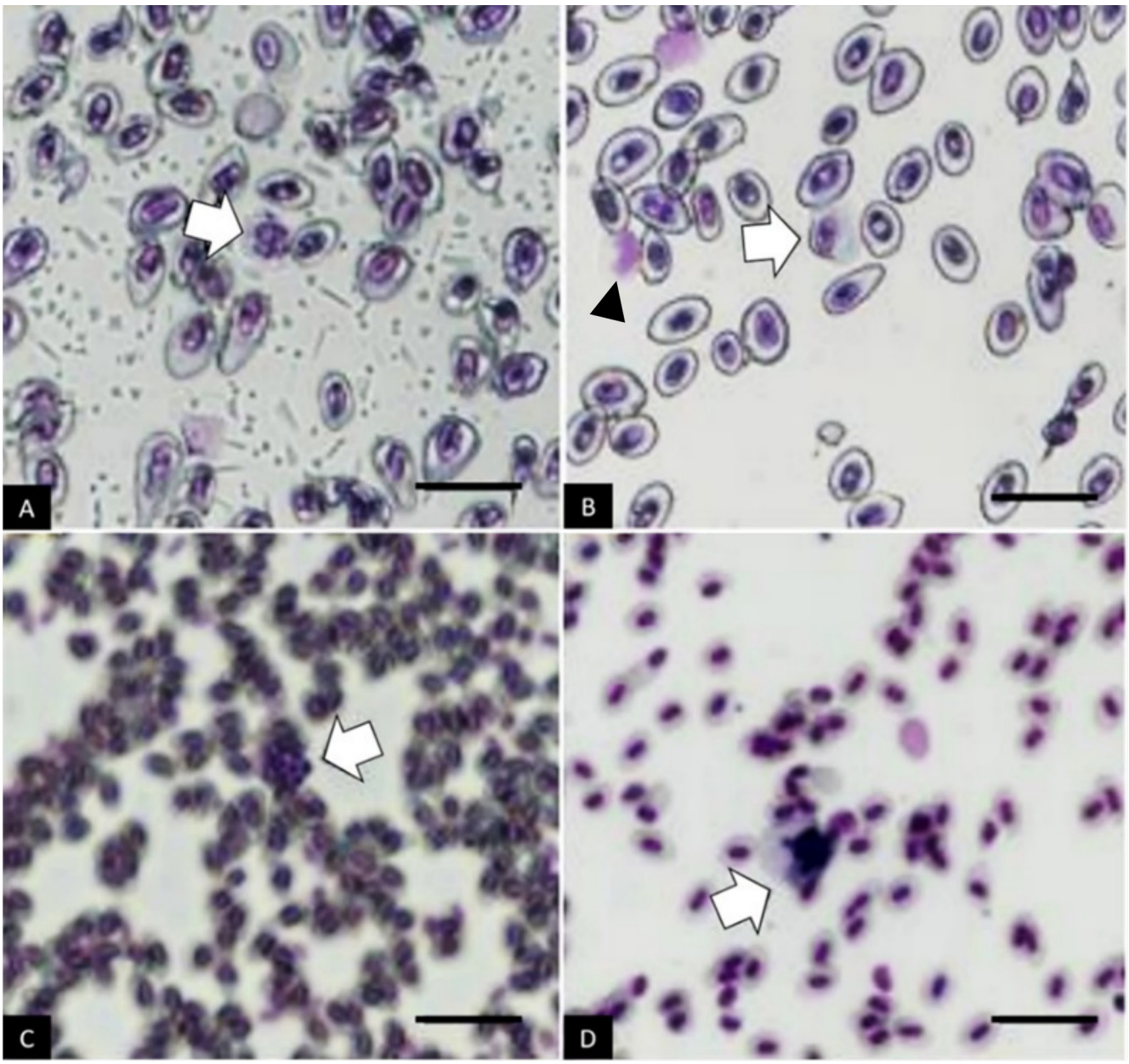

FIGURE 6 | Blood smears from different treatments in the in vivo administration of CsA experiment, submitted for white blood cell count. The white arrow indicates: **A**. Granulocyte. **B**. Eosinophil; the black arrowhead indicates a lymphocyte. **C**. Neutrophil. **D**. Monocyte. 200 µm scale.

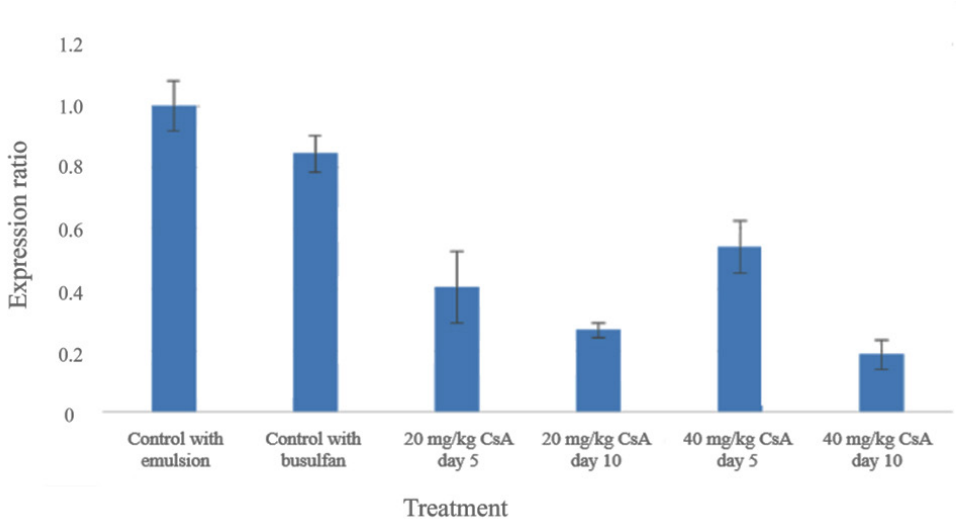

FIGURE 7 | Graph of relative expression of *interleukin-1*β in samples: control treated with emulsion 10 days after application, control treated with busulfan, treatment with CsA at 20 mg/kg five days after application, treatment with CsA at 20 mg/kg ten days after application, treatment with CsA at 40 mg/kg five days after application, and treatment with CsA at 40 mg/kg ten days after application.

DISCUSSION

Combining chromosome manipulation, busulfan-based chemical sterilization, and high-temperature treatment successfully sterilized triploid male *A. altiparanae* recipients, generating germ cell-free testes to receive transplanted germ cells. De Siqueira-Silva *et al.* (2015) demonstrated the effectiveness of the antineoplastic drug busulfan in achieving endogenous depletion of adult diploid *A. altiparanae*. Researchers also assessed this method in other fish species, including *Oreochromis niloticus* (Lacerda *et al.*, 2010), *Odontesthes bonariensis* (Majhi *et al.*, 2009), *Odontesthes hatcheri* (Majhi *et al.*, 2009), and *Danio rerio* (Nóbrega *et al.*, 2010). Chemical sterilization in adult diploid *A. altiparanae* was not permanent, and the fish returned to endogenous gametogenesis (de Siqueira-Silva *et al.*, 2015). Triploidization was used to sterilize *A. altiparanae* (Adamov *et al.*, 2017); however, this method was ineffective in generating recipients with germ-cell-less gonads essential for transplantation in adult recipients. Similarly, hybridization combined with heat-shock-induced triploidization produced predominantly sterile hybrids of *A. altiparanae* and *A. fasciatus* (Piva *et al.*, 2018). As an alternative, chromosome manipulation combined with chemical sterilization offers a promising method for producing adult recipients of *A. altiparanae* for spermatogonial transplantation.

After busulfan treatment, regardless of whether CsA was administered, the gonads observed in the final CsA experiment were small and had reduced mass. They were often only present on one side of the intracoelomic cavity, leading to gonadal regression that may not be suitable for receiving transplanted cells. This may have occurred because juvenile triploids were used in chemical sterilization, which may have affected normal gonadal development. Using adult triploid *A. altiparanae* may help minimize this problem. Recently, a study reported that adult triploid hybrid recipients (female

Astyanax altiparanae crossed with male *Astyanax fasciatus*) showed gonads devoid of germ cells (López *et al*., 2024). These hybrids could represent an alternative model for evaluating CsA *in vivo*, eliminating the need for chemical sterilization. In addition, chemical sterilization with busulfan followed by CsA administration has resulted in a significant mortality rate. However, the combination of chromosome manipulation and chemical sterilization can be an alternative way to produce recipients with germ cell-less gonads, aiming for germ cell transplantation.

CsA is commonly used as an immunosuppressant to prevent transplant rejection (Lee *et al*., 2023), inhibiting T cell proliferation, and is associated with genetic instability by repression of genes that activate these cells, such as interleukins (Matsuda, Koyasu, 2000; Cilião *et al*., 2015), and also because CsA exhibits cytotoxic and genotoxic properties, leading to unwanted mutagenesis (Cilião *et al*., 2015). *In vitro* culture of *A. altiparanae* leukocytes treated with CsA showed the drug's immunosuppressive and cytotoxic effects by assessing cell viability and concentration. The highest CsA concentration used showed greater immunosuppression by decreasing leukocyte concentration and viability. The results were similar to those from *in vitro* leukocyte cultures treated with CsA from *Oncorhynchus mykiss* (Yoshinaga *et al*., 2021) and *Paralichthys olivaceus* (Xing *et al*., 2017). The treatment reduced leukocyte concentration compared with the control group at 24 and 48 h of culture.

In the *in vivo* study involving CsA, the head kidney showed no changes on the 5th day after administration. By the tenth day, the head kidney had a more gelatinous consistency and easily fell apart, preventing histological analysis. Consequently, the administered CsA dosages induced nephrotoxicity, which may account for approximately 40% of the lethality observed in the immunosuppressed fish. Malachite green disinfectant, commonly used in fish to treat and prevent parasitic, fungal, and bacterial infections, can cause persistent leukopenia (Witeska *et al*., 2013). This compound affects the hematopoietic tissue in the head kidney, reducing the frequency of these cells in the organ and resulting in anemia in *Cyprinus carpio* (Witeska *et al*., 2013). Such conditions indicate an immunosuppressive effect. Likewise, CsA administration in *A. altiparanae* may have affected hematopoietic tissues in the head kidney, contributing to anemia and leukocyte reduction. These effects have also been reported for other immunosuppressive agents in fish and humans (Azeez, 2021; Elazab *et al*., 2021; Pande *et al*., 2023).

On day 10, lymphocytes from the group receiving 40 mg/kg CsA showed a slight decrease in proportion, suggesting the immunosuppressant had a partial effect. In contrast, the proportion of lymphocytes in the control group that received the emulsion remained unchanged, indicating that the adaptive response of lymphocytes towards the emulsion vehicle may have been prolonged. The control group treated with busulfan at the beginning of the experiment had more than double the proportion of lymphocytes compared to the control group without busulfan. Busulfan has minimal impact on triggering immune-mediated rejection (Uchida *et al*., 2019). However, the high temperature of 35°C during chemical sterilization likely caused stress and increased glucocorticoid production in the fish. Increased glucocorticoids may mediate immunomodulation, resulting in a higher proportion of lymphocytes and altered leukocyte proportions. This possibility is supported by Tsai, Wang (1997), in which *Oreochromis mossambicus* were subjected to 32°C for weeks and showed increased levels of stress hormones, including glucocorticoids. The change in hematological

parameters between the control groups at the beginning of the experiment, caused by the temperature rise in the busulfan treatment, contradicts previous studies that associate fish acute stress with a decrease in lymphocytes (lymphopenia), and an increase in the proportion of neutrophils (neutrophilia), increasing the neutrophil-to-lymphocyte ratio (Davis *et al*., 2008; Grzelak *et al*., 2017). Neutrophils and lymphocytes typically account for 70 to 80% of all leukocytes in fish, and this proportion was generally observed in this study. However, the current study did not adhere to the established neutrophil-to-lymphocyte ratio, potentially due to stress throughout the experiment, which also appears unaffected by CsA administrations.

The analysis of leukocyte populations and immunomodulation, which is the focus of this study, intersects with research that primarily evaluates stress levels and immunostimulants in the animals involved. Jalali *et al*. (2009) investigated the hematological parameters of *Huso huso* when fed experimental diets. Fish fed a diet supplemented with immunostimulant algae showed a higher proportion of lymphocytes than those in the control group that received no supplements. Lymphocytes modulate the immune response and are considered the most sensitive cell type to acute stress in fish (Tsai, Wang, 1997; Jalali *et al*., 2009), and are directly linked to physiology and immunological homeostasis (Wendelaar Bonga, 1997). These cells can produce antibodies and interact with macrophages to activate the immune system. The increase in this cell type observed by Jalali *et al*. (2009) in the *Huso huso* control group also occurred in the present study, mainly in the control group injected with emulsion on day 5. The 40 mg/kg treatments on day 5 showed an increase in the proportion of lymphocytes compared with day 10 (65.77% *vs.* 51.4%).

In *A. altiparanae* recipients, *in vivo* CsA administration reduced relative *interleukin-1*β expression in the head kidneys; 20 and 40 mg/kg reduced it by 73% and 81%, respectively, on day 10 after CsA application. Although both concentrations showed about 40% mortality, suggesting similar nephrotoxicity, the high mortality rates observed in both CsA-treated groups may also reflect the cumulative physiological stress associated with triploidization and high-temperature exposure during busulfan treatment. Nevertheless, the 60% surviving recipients may still provide a sufficient yield for germline chimera production. Therefore, 40 mg/kg CsA reduced *interleukin-1*β expression more than 20 mg/kg, although notable toxic effects were observed. Despite the relatively small experimental groups and the high mortality rate after CsA administration, which limited the statistical power of the non-parametric tests used, this study provides a useful preliminary framework for developing an optimized immunosuppression protocol for fish germ cell transplantation.

Yoshinaga *et al*. (2021) studied immunosuppression in transplanted fish treated with CsA, using intracoelomic administration at 20 mg/kg and 40 mg/kg in grafted *O. mykiss* with allogeneic testes. The highest dose produced longer-lasting immunosuppression by prolonging inhibition of *interleukin-2* expression. These findings also support the CsA immunosuppressive effects in *A. altiparanae* recipients.

*Interleukin-1*β, a pro-inflammatory cytokine essential for T-cell activation and proliferation and a regulator of the innate immune system (Verburg-Van Kemenade *et al*., 2009), was used as a molecular marker of immunosuppression in fish under immunosuppressant treatments, as discussed by Elazab *et al*. (2021). *O. niloticus interleukin-1*β expression in the head kidney treated with cyclophosphamide was

reduced compared to the untreated group (Elazab *et al*., 2021). Cytokine expression may be regulated by internal fish factors, such as stress hormones, due to the close connection between the neuroendocrine and immune systems (Harris, Bird, 2000). Castillo *et al*. (2009) reported that *interleukin-1*β expression is repressed by the stress-related hormones adrenocorticotropic hormone, adrenaline, and cortisol in cultures of hematopoietic cells from the *Sparus aurata* head kidney. This may lead to differences in *interleukin-1*β expression among individuals in the experimental groups.

The combination of chromosome manipulation and chemical sterilization with busulfan, at elevated temperatures, effectively depleted germ cells in male juvenile triploid *A. altiparanae* recipients, thereby creating available germinal space for transplantation. Furthermore, CsA administration promoted immunosuppressive effects while reducing *interleukin-1*β expression; however, notable hematological and renal side effects were also observed. No changes were observed in the cellular structure of the head kidney on the 5th day after administration, but the consistency was altered on the 10th day after administration of CsA. No evident gonadal alterations associated with CsA administration were observed.

This work established immunosuppression procedures in male juvenile triploid *A. altiparanae* sterilized with busulfan, providing a model fish to evaluate preliminary immunosuppressive effects. The methodology may contribute to generating germ cell recipients for further interspecific transplantation in Neotropical fish, potentially reducing cell rejection and improving the efficiency of future germline chimera production, supporting endangered ichthyofauna conservation actions.

ACKNOWLEDGMENTS

The authors acknowledge ICMBio/CEPTA for providing structural and human support.

REFERENCES

- **Adamov NSM, Nascimento NF, Maciel ECS, Pereira-Santos M, Senhorini JA, Calado LL *et al*.** Triploid induction in the yellowtail tetra, *Astyanax altiparanae*, using temperature shock: tools for conservation and aquaculture. J World Aquac Soc. 2017; 48(5):741–50. https://doi.org/10.1111/JWAS.12390
- **Azeez SH.** Impact of cyclosporine A on white blood cell counts and some physiological parameters in renal transplanted patients: a case control study. Int J Med Investig. 2021; 10(1):111–19. https://dor.isc.ac/dor/20.1001.1.23222913.2021.10.1.10.4
- **Brinster RL, Avarbock MR.** Germline transmission of donor haplotype following spermatogonial transplantation. PNAS. 1994; 91(24):11303–07. https://doi.org/10.1073/pnas.91.24.11303
- **Brinster CJ, Ryu BY, Avarbock MR, Karagenc L, Brinster RL, Orwig KE.** Restoration of fertility by germ cell transplantation requires effective recipient preparation. Biol Reprod. 2003; 69(2):412–20. https://doi.org/10.1095/biolreprod.103.016519
- **Castillo J, Teles M, Mackenzie S, Tort L.** Stress-related hormones modulate cytokine expression in the head kidney of gilthead seabream (*Sparus aurata*). Fish Shellfish Immunol. 2009; 27(3):493–99. https://doi.org/10.1016/j.fsi.2009.06.021

- **Chen X, Liang M, Wang D.** Progress on the study of the mechanism of busulfan cytotoxicity. Cytotechnology. 2018; 70:497–502. https://doi.org/10.1007/s10616-018-0189-5
- **Cilião HL, Ribeiro DL, Camargo-Godoy RB, Specian AF, Serpeloni JM, Cólus IM.** Cytotoxic and genotoxic effects of high concentrations of the immunosuppressive drugs cyclosporine and tacrolimus in MRC-5 cells. Exp Toxicol Pathol. 2015; 67(2):179–87. https://doi.org/10.1016/j.etp.2014.11.008
- **Davis AK, Maney DL, Maerz JC.** The use of leukocyte profiles to measure stress in vertebrates: a review for ecologists. Funct Ecol. 2008; 22(5):760–72. https://doi.org/10.1111/j.1365-2435.2008.01467.x
- **Demirci H, Popovic S, Dittmayer C, Yilmaz DE, El-Shimy IA, Mülleder M *et al*.** Immunosuppression with cyclosporine versus tacrolimus shows distinctive nephrotoxicity profiles within renal compartments. Acta Physiol. 2024; 240(8):e14190. https://doi.org/10.1111/apha.14190
- **Elazab MFA, Younes AM, Gaafar AY, Abu-Bryka AZ, Abdel-Daim MM.** Immunosuppressive effect of cyclophosphamide in Nile tilapia (*Oreochromis niloticus*). Environ Sci Pollut Res Int. 2021; 28:20784–93. https://doi.org/10.1007/s11356-020-11893-8
- **Franěk R, Cheng Y, Fučíková M, Kašpar V, Xie X, Shah MA *et al*.** Who is the best surrogate for germ stem cell transplantation in fish? Aquaculture. 2022; 549:737759. https://doi.org/10.1016/j.aquaculture.2021.737759
- **Grzelak AK, Davis DJ, Caraker SM, Crim MJ, Spitsbergen JM, Wiedmeyer CE.** Stress leukogram induced by acute and chronic stress in zebrafish (*Danio rerio*). Comp Med. 2017; 67(3):263–69.
- **Harris J, Bird DJ.** Modulation of the fish immune system by hormones. Vet Immunol Immunopathol. 2000; 77(3–4):163–76. https://doi.org/10.1016/s0165-2427(00)00235-x
- **Jalali MA, Ahmadifar E, Sudagar M, Takami GA.** Growth efficiency, body composition, survival and haematological changes in great sturgeon (*Huso huso* Linnaeus, 1758) juveniles fed diets supplemented with different levels of Ergosan. Aquac Res. 2009; 40(7):804–09. https://doi.org/10.1111/j.1365-2109.2009.02166.x
- **Lacerda SM, Batlouni SR, Costa GM, Segatelli TM, Quirino BR, Queiroz BM *et al*.** A new and fast technique to generate offspring after germ cells transplantation in adult fish: the Nile tilapia (*Oreochromis niloticus*) model. PLoS ONE. 2010; 5(5):e10740. https://doi.org/10.1371/journal.pone.0010740
- **Lee H, Myoung H, Kim SM.** Review of two immunosuppressants: tacrolimus and cyclosporine. J Korean Assoc Oral Maxillofac Surg. 2023; 49(6):311–23. https://doi.org/10.5125/jkaoms.2023.49.6.311
- **Levy-Pereira N, Carriero MM, Yasui GS, Meira CM, Sousa RLM, Maia AAM *et al*.** Effects of triploid induction on innate immunity and hematology in *Astyanax altiparanae*. Fish Shellfish Immunol. 2021; 116:12–18. https://doi.org/10.1016/j.fsi.2021.05.004
- **Li M, Hong N, Xu H, Song J, Hong Y.** Germline replacement by blastula cell transplantation in the fish medaka. Sci Rep. 2016; 6:(29658). https://doi.org/10.1038/srep29658
- **López LS, Monzani PS, Carvalho GB, de Siqueira Silva DH, Vianna NC, Yasui GS *et al*.** Cryopreservation and transplantation of spermatogonia stem cells in piracanjuba *Brycon orbignyanus* (Characiformes: Characidae), an endangered fish species. Fish Physiol Biochem. 2024; 50:2117–35. https://doi.org/10.1007/s10695-024-01406-6
- **López LS, Bertolini RM, Monzani P, Carvalho GB, Siqueira-Silva DH, Senhorini JA *et al*.** Spermatogenesis depletion in diploid and triploid yellowtail tetra (*Astyanax altiparanae*) for germ cell transplantation. Zygote. 2026; 34(2):62–75. https://doi.org/10.1017/S0967199426100380
- **Majhi SK, Hattori RS, Yokota M, Watanabe S, Strüssmann CA.** Germ cell transplantation using sexually competent fish: an approach for rapid propagation of endangered and valuable germlines. PLoS ONE. 2009; 4(7):e6132. https://doi.org/10.1371/journal.pone.0006132
- **Matsuda S, Koyasu S.** Mechanisms of action of cyclosporine. Immunopharmacology. 2000; 47(2–3):119–25. https://doi.org/10.1016/s0162-3109(00)00192-2

- **Nagasawa K, Ishida M, Octavera A, Kusano K, Kezuka F, Kitano T *et al*.** Novel method for mass producing genetically sterile fish from surrogate broodstock via spermatogonial transplantation. Biol Reprod. 2019; 100(2):535–46. https://doi.org/10.1093/biolre/ioy204
- **Nóbrega RH, Greebe CD, van de Kant H, Bogerd J, de França LR, Schulz RW.** Spermatogonial stem cell niche and spermatogonial stem cell transplantation in zebrafish. PLoS ONE. 2010; 5(9):e12808. https://doi.org/10.1371/journal.pone.0012808
- **Okutsu T, Suzuki K, Takeuchi Y, Takeuchi T, Yoshizaki G.** Testicular germ cells can colonize sexually undifferentiated embryonic gonad and produce functional eggs in fish. PNAS. 2006; 103(8):2725–29. https://doi.org/10.1073/pnas.0509218103
- **Pande A, Kumar A, Krishnani H, Acharya S, Shukla S.** Recent advances in the management of microangiopathic hemolytic anemias (MAHA): a narrative review. Cureus. 2023; 15(10):e47196. https://doi.org/10.7759/cureus.47196
- **Piva LH, Siqueira-Silva DH, Goes CAG, Fujimoto T, Saito T, Dragone LV *et al*.** Triploid or hybrid tetra: Which is the ideal sterile host for surrogate technology? Theriogenology. 2018; 108:239–44. https://doi.org/10.1016/j.theriogenology.2017.12.013
- **Ranzani-Paiva MJT, Godinho HM.** Hematological studies in curimbatá, *Prochilodus scrofa* Steindachner, 1881 (Osteichthyes, Cypriniformes, Prochilodontidae). Bol Inst Pesca. 1985; 12(2):25–35.
- **Ryu JH, Xu L, Wong TT.** Advantages, factors, obstacles, potential solutions, and recent advances of fish germ cell transplantation for aquaculture – A practical review. Animals. 2022; 12(4):423. https://doi.org/10.3390/ani12040423
- **Saito T, Goto-Kazeto R, Arai K, Yamaha E.** Xenogenesis in teleost fish through generation of germ-line chimeras by single primordial germ cell transplantation. Biol Reprod. 2008; 78(1):159–66. https://doi.org/10.1095/biolreprod.107.060038
- **Sarrafzadeh O, Rabbani H, Talebi A, Banaem HU.** Selection of the best features for leukocytes classification in blood smear microscopic images Proc SPIE. 2014; 9041. https://doi.org/10.1117/12.2043605
- **de Siqueira-Silva DH, Silva AP, Ninhaus-Silveira A, Veríssimo-Silveira R.** The effects of temperature and busulfan (Myleran) on the yellowtail tetra *Astyanax altiparanae* (Pisces, Characiformes) spermatogenesis. Theriogenology. 2015; 84(6):1033–42. https://doi.org/10.1016/j.theriogenology.2015.06.004
- **Takeuchi Y, Yoshizaki G, Takeuchi T.** Generation of live fry from intraperitoneally transplanted primordial germ cells in rainbow trout. Biol Reprod. 2003; 69(4):1142–49. https://doi.org/10.1095/biolreprod.103.017624
- **Tsai CL, Wang LH.** Effects of thermal acclimation on the neurotransmitters, serotonin and norepinephrine in the discrete brain of male and female tilapia, *Oreochromis mossambicus*. Neurosci Lett. 1997; 233(2–3):77–80. https://doi.org/10.1016/s0304-3940(97)00618-6
- **Uchida N, Nassehi T, Drysdale CM, Gamer J, Yapundich M, Bonifacino AC *et al*.** Busulfan combined with immunosuppression allows efficient engraftment of gene-modified cells in a rhesus macaque model. Mol Ther. 2019; 27(9):1586–96. https://doi.org/10.1016/j.ymthe.2019.05.022
- **Verburg–Van Kemenade BML, Stolte EH, Metz JR, Chadzinska M.** Neuroendocrine-immune interactions in teleost fish. In: Bernier NJ, Van Der Kraak G, Farrell AP, Brauner CJ, editors. Fish neuroendocrinology. San Diego, CA: Academic Press; 2009. p:313–64. https://doi.org/10.1016/S1546-5098(09)28007-1
- **Xavier PLP, Senhorini JA, Pereira-Santos M, Fujimoto T, Shimoda E, Silva LA *et al*.** A flow cytometry protocol to estimate DNA content in the yellowtail tetra *Astyanax altiparanae*. Front Genet. 2017; 8:131. https://doi.org/10.3389/fgene.2017.00131
- **Xing J, Xiao Y, Tang X, Sheng X, Zhan W.** Inhibition of cyclosporine A or rapamycin on T lymphocyte counts and the influence on the immune responses of B lymphocytes in flounder (*Paralichthys olivaceus*). Fish Shellfish Immunol. 2017; 66:78–85. https://doi.org/10.1016/j.fsi.2017.05.017
- **Wendelaar Bonga SE.** The stress response in fish. Physiol Rev. 1997; 77(3):591–625. https://doi.org/10.1152/physrev.1997.77.3.591

- **Witeska M, Kondera E, Belniak N.** Hematological and hematopoietic changes induced by formaldehyde and malachite green in common carp (*Cyprinus carpio* L.). Zool Ecol. 2013; 23(3):245–51. https://doi.org/10.1080/21658005.2013.821790
- **Yoshinaga TT, Kfoury Júnior JR, Butzge AJ, Olio RL, Hernandez-Blazquez FJ, Oliveira Carreira AC *et al*.** Testicular subcutaneous allografting followed by immunosuppressive treatment promotes maintenance of spermatogonial cells in rainbow trout (*Oncorhynchus mykiss*). Fish Shellfish Immunol. 2021; 112:108–15. https://doi.org/10.1016/j.fsi.2021.03.002
- **Zohni K, Zhang X, Tan SL, Chan P, Nagano MC.** The efficiency of male fertility restoration is dependent on the recovery kinetics of spermatogonial stem cells after cytotoxic treatment with busulfan in mice. Hum Reprod. 2012; 27(1):44–53. https://doi.org/10.1093/humrep/der357

AUTHORS' CONTRIBUTION

Giselle P. Pessoa: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Visualization, Writing-original draft, Writing-review and editing.

Lucia S. López: Conceptualization, Formal analysis, Investigation, Methodology, Visualization, Writing-review and editing.

Amanda P. S. Silva: Formal analysis, Investigation, Methodology, Visualization, Writing-review and editing.

Silvio C. A. Santos: Funding acquisition, Project administration, Writing-review and editing.

George S. Yasui: Funding acquisition, Project administration, Writing-review and editing.

José A. Senhorini: Conceptualization, Methodology, Supervision, Visualization, Writing-review and editing.

Paulo S. Monzani: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Supervision, Visualization, Writing-original draft, Writing-review and editing.

FUNDING INFORMATION

This work was supported by the AES Tietê (Research & Development Project #0064–1062/2020), São Paulo Research Foundation - FAPESP (Award Grant #2016/16386–3), and ICMBio/CEPTA for providing both structural and human support for the project.

ETHICAL STATEMENT

The experiments were conducted in accordance with the guidelines established by the Animal Ethics Committee from CEPTA/ICMBio (CEUA/CEPTA Nº 0064–1062/2020).

DATA AVAILABILITY STATEMENT

The authors confirm that the data supporting the findings of this study are available within the article and its supplementary material.

AI STATEMENT

The authors used Claude (Anthropic) as an AI-assisted tool for grammar correction and language editing of this manuscript.

COMPETING INTERESTS

The authors declare no competing interests.

SUPPLEMENTARY MATERIAL

Supplementary Material File

PEER REVIEW REPORT

Reviewers:

Anonymous reviewer #1

Anonymous reviewer #2

The peer review report is available at: Neotropical Ichthyology

HOW TO CITE THIS ARTICLE

- **Pessoa GP, López LS, Silva APS, Santos SCA, Yasui GS, Senhorini JA, Monzani PS.** Cyclosporine A administration in chemically sterilized triploid *Astyanax altiparanae* to prevent cell rejection for future interspecific transplantation of fish germ cells. Neotrop Ichthyol. 2026; 24(4):e250213. https://doi.org/10.1590/1982-0224-202-0213

Neotropical Ichthyology

OPEN ACCESS

This is an open access article under the terms of the Creative Commons Attribution License, which permits use, distribution and reproduction in any medium, provided the original work is properly cited.

Distributed under
Creative Commons CC-BY 4.0

© 2026 The Authors.
Published by SBI

Official Journal of the
Sociedade Brasileira de Ictiologia

SBI