



Long-term dexamethasone treatment increases the engraftment efficiency of human breast cancer cells in adult zebrafish



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ABSTRACT

The host immune system tends to reject xenogenic-implanted cells making tumor development in adult host animal models difficult. Immune system suppression is used for successful xenotransplantation of human cancer cells in many animal models. The studies of cancer development processes *in vivo* offer opportunities to understand cancer biology and discover new therapeutic strategies. In this context, zebrafish is a model that has been widely applied in the study of human diseases, such as cancer. However, the long-term immunosuppression of these adult zebrafish is still under study as a xenograft animal model for human cancer. This work aimed to evaluate the effects of 21 days of (long-term) exposure of dexamethasone in zebrafish-transplanted with MGSO-3 cells, human breast tumor cell line. Our results show that the animals, while kept on dexamethasone treatment, remained with a 50% reduction in the number of peripheral lymphocytes. *In vitro* data demonstrated that up to 7 days of dexamethasone treatment did not alter the morphology, proliferation, or viability of MGSO-3 cells. The animals that received a prolonged dexamethasone treatment allowed the engraftment of tumor cells in 100% of the zebrafish tested. These animals also showed tumor progression over 21 days. The experimental group that received only previous exposure to dexamethasone had their tumors regressed after 14 days. In conclusion, the prolonged use of dexamethasone in zebrafish showed a potential strategy for *in vivo* monitoring of xenograft tumor growth for development studies, as well as in anticancer drug discovery.

1. Introduction

Breast cancer is a worldwide disease that affects the public health of all nations [1,2]. According to GLOBOCAN, Global Cancer Observatory, breast cancer is the most commonly diagnosed among females and is the fourth leading cause of cancer death [2]. Anticancer therapies have improved treatments, but these therapies do not fulfill their function, especially if the disease is at an advanced stage [3].

Precision oncology has revolutionized in the medical field due to the possibility of increasing the effectiveness of treatments. The focus of precision oncology is to optimize treatment for an individual patient rather

than a group. This individual study can guide clinical treatment decisions and drive the use of some drugs that modulate the specific target tissue activity [4,5]. Studies using patient-derived cancer cell xenotransplants have demonstrated the feasibility of using zebrafish as a model for prognosis and response to drugs to identify the ideal therapy for an individual patient [6].

Attractive features make zebrafish an excellent model for testing anticancer therapies. Zebrafish are an inexpensive animal to keep in the laboratory, develop *ex vivo* rapidly, and can be kept in small volumes of water [6–9]. Several studies have demonstrated the ability of this model to support xenogenic transplants of human origin, allowing the direct

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study of human cells without the expenditure of genetically modified mice [10–14].

In zebrafish embryos, the immune system is immature, which does not require the use of immunosuppressive agents in tumor development [15,16]. After 21 days of fertilization, the zebrafish develops a fully functional innate and adaptive immune system and transplanted cancer cells are rejected. This is considered a limitation because many of the tumor types predominantly occur in adults [17]. Xenotransplantation in juvenile and adult zebrafish seeks to overcome this restriction and opens up new avenues to study the development and metabolism of adult cancers [14].

The challenge of generating immune-deficient adult zebrafish has been overcome through irradiation and genetic mutation. These immunosuppression types promote vulnerability to spontaneous infection leading to death within a couple of days [11,18]. Another method of achieving immunosuppression is to expose fish to dexamethasone [19]. Some studies demonstrated successful xenotransplants of tumor cells for few days in zebrafish when the animals were exposed to dexamethasone for two days [20–22]. We hypothesized that prolonged exposure of dexamethasone might allow the maintenance and development of tumor cells in the fish organism.

This present work aimed to evaluate the influence of dexamethasone on the zebrafish immune system for 21 days. It was observed that the maintenance of dexamethasone treatment reduces the number of peripheral lymphocytes allowing the efficient engraftment of MGSO-3 cells for 21 days. This study may bring relevant knowledge about the use of adult zebrafish as a model to understand the biology of human cancer and for drug discovery.

2. Material and methods

2.1. Animals and biometrics

The adult zebrafish females present a total length and body weight of 3.70 ± 0.60 cm and $0.46 \text{ g} \pm 0.1$, respectively. The procedures were performed according to the animal health care guidelines. All procedures were approved by the Ethics Committee on Animal Experimentation (CEUA - UFMG) and received the protocol number 375/2017.

2.3. Dexamethasone exposure

Before the experiments, fish were acclimated under laboratory conditions for 3 weeks in a 10 L glass aquarium with dechlorinated water (28 °C) and a 12/12 h light/dark photoperiod. All the treatments received forced aeration through a semi-static system. A total of 84 adult female specimens of zebrafish were randomly assigned in 3 aquariums of 10 L filled with dechlorinated water. Each tank housed 28 animals and each of them received a type of treatment: Group 1 did not receive the addition of dexamethasone and therefore, it was considered the control group. The other two groups received dexamethasone (dexamethasone-water soluble from Sigma-Aldrich) in the concentration of 10 mg/L [21]. In Group 2, the animals were exposed to dexamethasone for 48 h (short DEX group) [20,23]. The zebrafish of group 3 remained exposed to dexamethasone throughout the experiment, representing the long DEX group. Every 48 h, 50% of the total water volume of the aquarium was renewed and the dexamethasone concentration was replenished. At each experimental time (1, 3, 5, and 7 days), seven animals were removed from each treatment, anesthetized with tricaine (0.1 mg/mL) and blood samples were obtained from these animals. After obtaining the blood, the animals were euthanized in a water and ice bath. On the first day of analysis, the animals belonging to group 2 and 3 were exposed to dexamethasone. However, in the following times, only the animals belonging to long DEX were kept on dexamethasone (Fig. 1a). The water temperature was maintained at 32 °C throughout the experiment.

2.4. Lymphocytes quantification by flow cytometry

The immunosuppressive action of dexamethasone was evaluated by flow cytometry (Becton Dickinson FACSCanto™) in zebrafish exposed to dexamethasone. The peripheral blood was obtained from the caudal vein using an ultrafine insulin syringe (BD) filled with 10 µL of 0.25 M ethylenediaminetetraacetic acid (EDTA) at pH 6.8. 10 µL of each sample were resuspended in 1 mL of phosphate-buffered saline (PBS, pH 7.4; Thermo Fisher Scientific) and immediately analyzed by BD FACSCanto™. The cell population was selected using the forward-scattered (FSC) x side-scattered (SSC). An amount of 100 000 cells were analyzed in each animal. The data were evaluated by the Software FlowJo (FlowJo, LLC) [24,25].

2.5. Cell culture

The breast cancer cell line MGSO-3 was established by our research group from breast tumors fragments, as previously described by Correa et al. [26]. The cell lines were cultured in Dulbecco modified Eagle's medium (DMEM, Gibco, Waltham, MA, USA) with 10% Fetal Bovine serum, 200 mM glutamine, 100 µg/mL streptomycin, and 100 U/mL penicillin. The cultures were kept in a humidified incubator with 5% CO₂ at 37 °C.

2.6. Cellular viability

The viability of MGSO-3 cells was evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT; ThermoFisher, Waltham, MA, USA), as previously described [27]. This assay is based on a reduction of tetrazolium salt formazan crystals by living cells [28]. Briefly, 1×10^4 cells were seeded into 24-well plates per 24 h. Subsequently, the cells were treated with 0.01, 0.1, and 1 mg/mL of dexamethasone. The negative control received only PBS. After treatment, the cell groups were evaluated at the end of four times (1, 3, 5, and 7 days). The medium was then removed and a solution containing 210 µL DMEM medium plus 170 µL MTT (5 mg/mL) per well was added. After 2 h, the formazan crystals were visualized under a light microscope and then dissolved in 210 µL of 10% SDS in 0.01 M HCl (Sigma-Aldrich, St. Louis, MO, USA). After overnight incubation, the optical density (O.D) of the solution was determined by measuring the absorbance at 595 nm using a plate reader. The experiment was performed in biological triplicate.

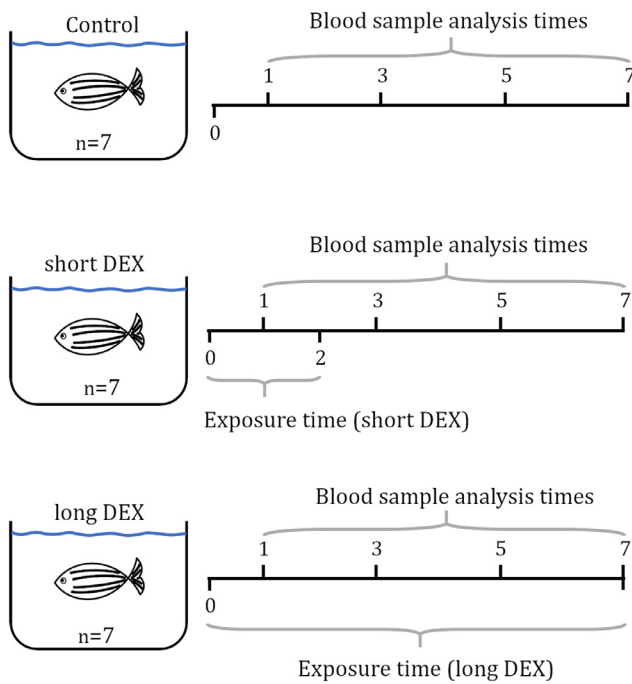
2.7. Growth curve assay

The growth of MGSO-3 cells when cultured in a solution containing dexamethasone (Sigma Aldrich) (1 mg/mL) was evaluated by testing the cell growth curve. A total of 1.3×10^4 MGSO-3 cells (per well) were seeded in 6-well plates for 24 h. Subsequently, the cells were treated with 1 mg/mL of dexamethasone (maximum treatment dose stipulated in this study) and 1x PBS was used as a negative control. The cells were kept at 37 °C in a humidified atmosphere of 5% CO₂. The culture medium was changed every two days. After treatment, cell groups were counted using a hemocytometer after 1, 3, 5, and 7 days. The experiment was performed in biological triplicates [29].

2.8. Xenograft in adult zebrafish

The MGSO-3 cells were trypsinized and labeled with 5 µL of Cell-Mask (ThermoFisher) at 10 nmol/L [30]. After labeling, the tumor cells were embedded in 7 µL of Matrigel (ECM Gel from Engelbreth-Holm-Swarm murine sarcoma – Sigma) and injected into the coelomic cavity of the fish (20 µL containing 3 000 cells) [18,31,32]. A total of 45 female fish were randomly assigned to 3 experimental groups: Control, short DEX and long DEX (15 animals per group). The zebrafish from the long DEX group started the dexamethasone treatment together with

a Experimental design



b Quantification of circulating blood cells

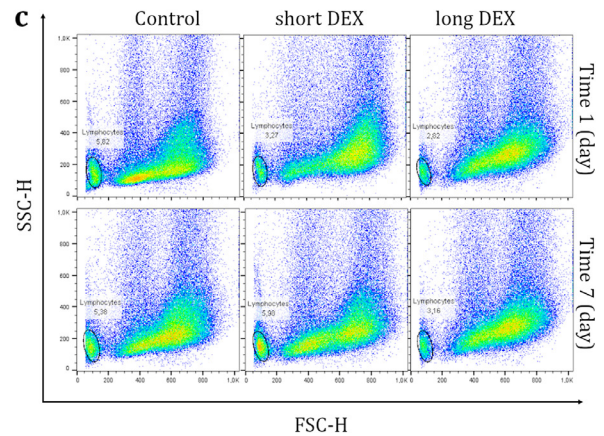
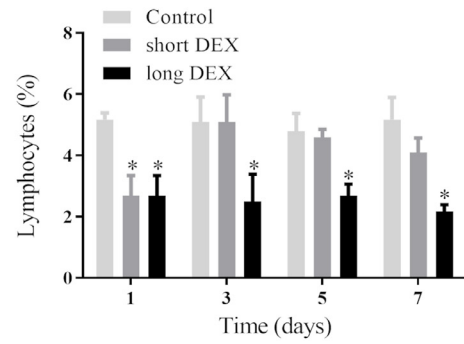


Fig. 1. Long-term treatment with dexamethasone decreases the number of lymphocytes. (a) Scheme of the experimental design. The animals received dexamethasone in the concentration of 10 mg/L. One group was exposed to dexamethasone for 48 h (short DEX) and another group remained exposed to dexamethasone throughout the experiment (long DEX). (b) The percentage of lymphocytes from total blood was performed on 1, 3, 5, and 7 days. Asterisks denote statistical significance ($p < 0.05$). (c) Representative graphs of FSC vs SSC data used to quantify the percentage of lymphocytes by flow cytometry.

group 2. However, animals of group 3 remained exposed to dexamethasone throughout the experiment (21 days). After the insertion of tumor cells in the zebrafish cell cavity, the aquarium's temperature were adjusted to 32 °C to improve the development of human tumor cells in the fish organism [32]. Every 48 h, 50% of the total water volume of the aquarium was renewed and the dexamethasone concentration was replenished. The animals were anesthetized with tricaine (0.1 mg/mL) at each experimental time and after capturing the images, they were returned to their respective treatments. The success of xenotransplantation was assessed by non-invasive imaging and measurement of the fluorescence area.

2.9. Imaging

The images were acquired using Zeiss AxioZoom.V16. The color balance was corrected and adjusted for display levels. All treatments or specimens under analysis were treated identically. For fluorescence imaging, DsRed (for CellMask) filters were selected and the development of the tumor was evaluated 7, 14, and 21 dpi. The fluorescence area was determined using ImageJ software (National Institutes of Health) [33].

2.10. Statistical analysis

Statistical analyses were performed by GraphPad Prism 5 software. For the cell viability, the One-way ANOVA followed by the Tukey post-test was used. Growth curve assay, lymphocytes quantification, and *in vivo* assay were analyzed by a One-way ANOVA followed by the Bonferroni post-test. The reported values were expressed as mean ± standard

deviation (S.D.) and results with $p < 0.05$ were considered statistically significant.

3. Results

3.1. Lymphocytes quantification by flow cytometry

Lymphocyte and granulocyte quantification were performed using flow cytometry to confirm the immunosuppression of the animals. On the first day of analysis, both groups, short DEX and long DEX, were exposed to dexamethasone (10 mg/L), but from day 2 the short DEX animals were removed from dexamethasone (Fig. 1a). The result shows a decrease in the number of lymphocytes in the long DEX group in all the time points studied. In contrast, a decrease in the number of lymphocytes was observed only on the first day in the short DEX group. After 3 days, the lymphocytes returned to the baseline level (Fig. 1b). The granulocytes remained stable, showing no change in their frequency throughout the experiment (Fig. 1b). Flow SSC vs FSC graphs represent the lymphocyte quantification in two treatments (short and long DEX) over time.

3.2. Dexamethasone treatment did not alter proliferation or metabolism of MGSO-3 cells

The proliferative behavior of the breast cancer cell line MGSO-3 exposed to different concentrations of dexamethasone (0.01, 0.1 e 1 mg/mL) was compared to the control cells. The results show that the dexamethasone treatment only alters the MTT metabolism with the lowest dose on the first day of treatment ($p > 0.05$) but did not alter cell

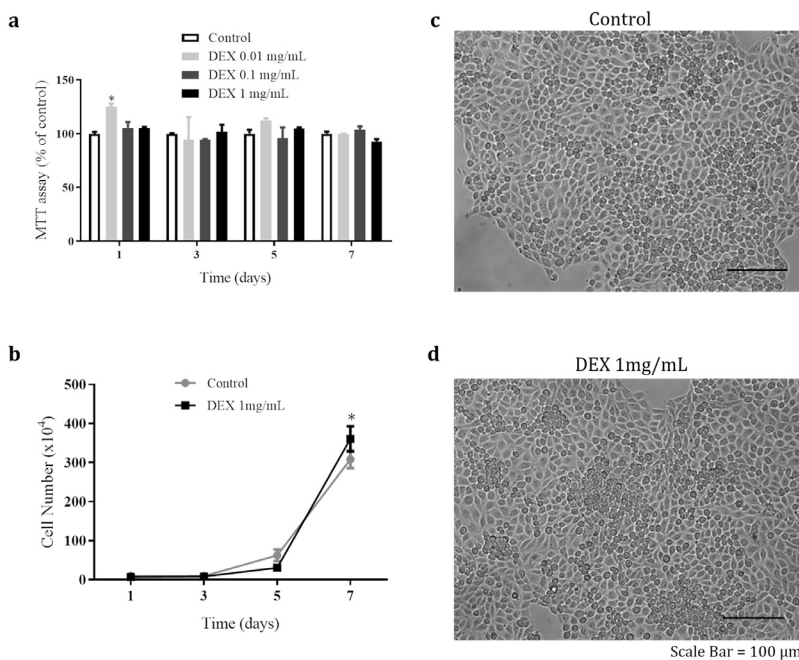


Fig. 2. Dexamethasone treatment did not alter MTT metabolism and cell proliferation of MGSO-3 cells. (a) The cytotoxic effect of dexamethasone was verified by the MTT assay. The optical density units (O.D.) measured at 595 nm were normalized as a percentage of control. (b) Cells were counted by the Neubauer chamber every 48 h to build the growth curves. (c and d) Images of MGSO-3 cell cultures not exposed and exposed to dexamethasone. Asterisks denote statistical significance ($p < 0.05$). The experiment was performed in biological triplicate ($n = 3$). (c and d) Brightfield images show that dexamethasone did not alter the morphology of the cells.

MTT metabolism in any of the three time points analyzed ($p < 0.05$), thus not presenting any cytotoxic effect in this cell line in most of the doses and times tested (Fig. 2a). Also, MGSO-3 cells, when exposed to dexamethasone, showed a similar proliferation curve compared to the control cells (Fig. 2b). The MGSO-3 cells reached a complete confluence simultaneously compared to control cells (Fig. 2c and 2d). No morphological changes were observed under the dexamethasone treatment.

3.3. Tumor engraftment in adult zebrafish

The tumor cell implant assay in adult zebrafish revealed that regardless of the dexamethasone treatment, 33% of the animals showed fluorescent cells in the same region where the tumor cells were injected after 7 dpi (days post-injection). After 14 and 21 dpi, 100% of the animals in the long DEX group were engrafted with the tumor cell line, but in the short DEX group, the percentage of animals engrafted with tumors did not change after 14 and 21 dpi (33%). Also, the measurement of the fluorescence area indicated an increase in tumor proliferation over time in the animals in the long DEX group and a decrease in the fluorescence area in the short DEX experimental group (Fig. 3a, b and c). The results showed that immunosuppression of zebrafish for more than 18 days had 6.6% of lethality ($n = 1/15$) (Fig. 3d).

4. Discussion

This study investigated the influence of long-term dexamethasone treatment on the engraftment efficiency of a tumor cell line in zebrafish. Our results demonstrated that dexamethasone affected the early phase of the tumor engraftment. Still, only animals that remained on long-term the dexamethasone treatment were successful in keeping the xenotransplants. It was shown that dexamethasone has low toxicity on tumor cells and animals. The partial immunosuppression demonstrated in the quantification of lymphocytes was enough to allow xenografts and prevent the animals' death.

Irradiation is also used to suppress the immune system of adult fish. However, a single dose of irradiation can lead to high lethality rates, depending on the dosage [11]. In addition to high mortality, the immune system of the irradiated zebrafish recovered after a few weeks, which may cause tumor rejection to remain a concern. Another strategy

to generate immuno-deficient zebrafish models is through gene mutation [18]. The generation of zebrafish SCID (severe combined immunodeficiency) performed by Jung et al. [18] demonstrated success in the tumor cell growth in the zebrafish. Still, the animals were vulnerable to spontaneous infection leading to death within a couple of days. We demonstrated that the concentration of dexamethasone used presented low lethality and allowed the development of human tumor cells in the zebrafish organism through its immunosuppressive effects.

The reduction of the peripheral lymphocyte count in the animals exposed to dexamethasone seen in this work confirmed the immunosuppressive effects of this drug. This is supported by studies showing that dexamethasone can compromise the innate immune responses to intracellular pathogens by inhibiting the cytotoxic activity of human natural killer cells and some cytokines involved in an immune response, such as IL-2 and IL-12 [34,35].

Clinical studies have shown that immunosuppression by dexamethasone may affect the patient's antitumor immunity. Wong et al. [36] demonstrated that the use of dexamethasone in patients had profound effects on the effectiveness of cancer treatments resulting in lower patient survival. These findings reinforce our results and confirm dexamethasone as a powerful and effective immunosuppression drug.

Zebrafish xenograft studies allow scientists to visualize various aspects of tumor formation, such as angiogenesis, migration and metastasis [6,12,37]. The possibility of monitoring tumor development and the study of new drugs shows that zebrafish is suitable and reliable in cancer research. A rapid "in vivo" drug response in zebrafish can lead to an improvement in personalized medicine, increasing treatment effectiveness [38]. Studies have shown success in xenotransplantation of fluorescent human breast (MDA-435), sarcoma (HT1080) and brain tumors cells into the peritoneal cavity of zebrafish, which have been treated with the immunosuppressant dexamethasone [20–22]. These cell types mentioned above formed tumors at the injection site. Still, they were available in a slightly short time, suggesting that there may be a failure in the immunosuppression of these animals, not allowing long assays. Our results showed success in MGSO-3 xenotransplantation in a long-term experiment, allowing long-term metabolic and metastatic studies.

The growth curve assay and viability assay were carried out to verify the influence of dexamethasone on MGSO-3 tumor cell viability, and our results showed no change in cell number over time and toxicity effects when compared with the control group. These data reinforced

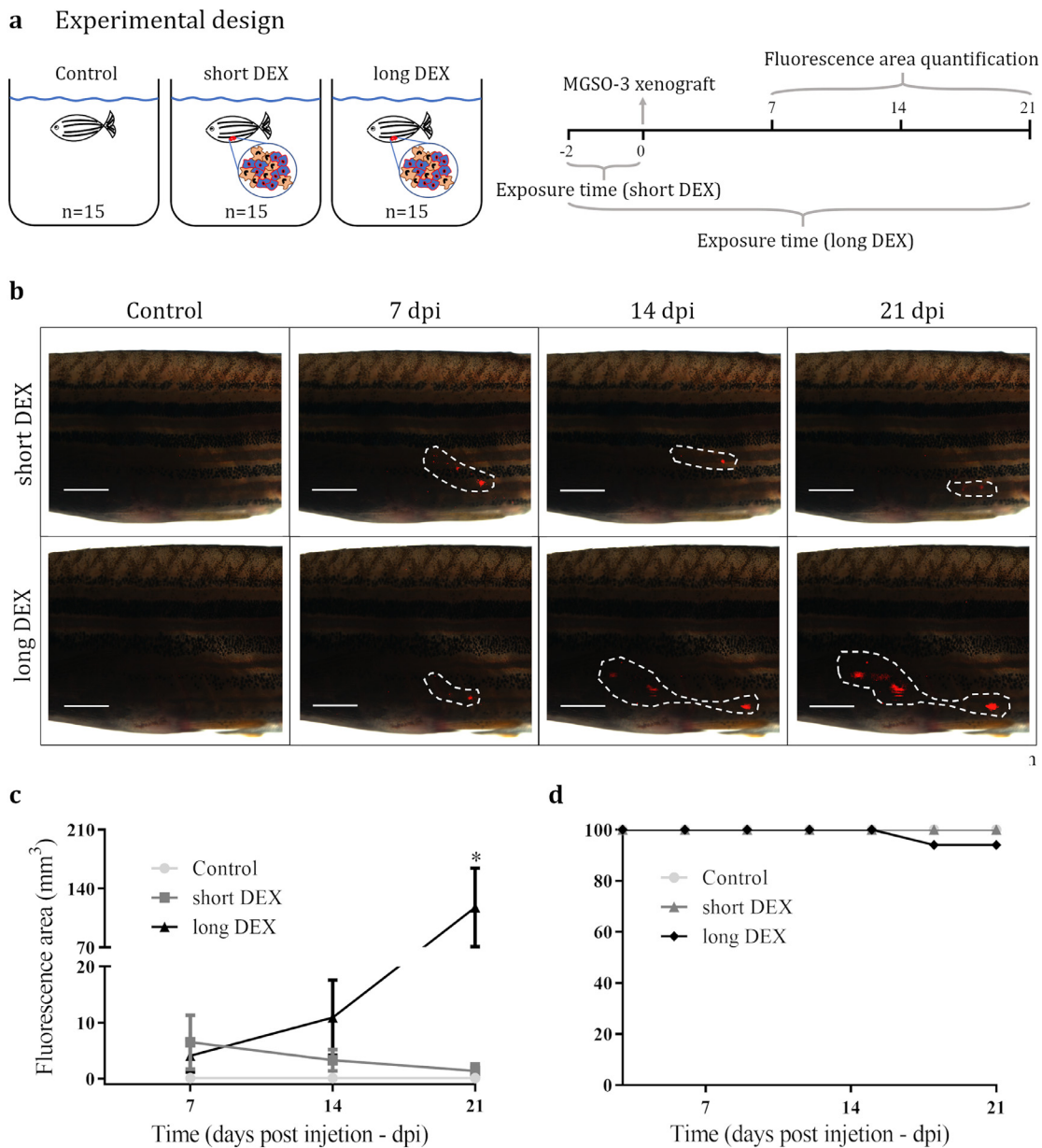


Fig. 3. Long-term dexamethasone treatment allows tumor growth for 21 days. (a) The breast cancer cells (MGSO-3) were labeled with Cell Mask, embedded in Matrigel and injected into the celomic cavity of the fish (20 μ L containing 3000 cells). Xenotransplantation of MGSO-3 cells in the celomic cavity was performed on animals exposed to short DEX and long DEX. (b) and (c) Fluorescence area measurements demonstrated that tumor cells developed better when animals were exposed to dexamethasone throughout the experiment. (d) Only one death was observed in the long DEX group. Values followed by different letters denote statistical significance ($p < 0.05$).

the safety of using dexamethasone on the viability of tumor cells. Another relevant aspect was that the morphological characteristics of the tumor cells did not change. MGSO-3 cells demonstrated a typical epithelial morphology of cuboids or polygonal cells growing in adherent monolayer as described previously by Correa et al. [26,27].

In conclusion, the long exposure to dexamethasone decreased the number of lymphocytes and this provided a suitable environment for the installation and growth of MGSO-3 tumor cells in zebrafish. Our study shows that dexamethasone treatment had no effects on MGSO-3 cell viability and demonstrates the potential use of long-term use of this drug for proper tumor engraftment in zebrafish for cancer research.

Declaration of Competing Interest

The authors declare that they have no competing interests.

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