QATAR UNIVERSITY

COLLEGE OF HEALTH SCIENCES

TESTING THE ANTICANCER EFFECT OF MATCHA USING ZEBRAFISH AS AN ANIMAL MODEL

BY

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A Thesis Submitted to

the College of Health Sciences

in Partial Fulfillment of the Requirements for the Degree of

Masters of Science in Human Nutrition

June 2023

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ABSTRACT

MOHAMED, SARA S., Masters of Science: June: [2023], Health Sciences

Title: Testing the Anticancer Effect of Matcha Using Zebrafish as an Animal Model.

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Cancer is the second leading cause of death worldwide. Triple-negative breast cancer (TNBC) patients show the poorest prognosis and survival and the highest metastasis prevalence among all breast cancer subtypes. Matcha (powder of Japanese green tea) has recently been associated with multiple health benefits, and *in vitro* studies showed the potential effect of matcha in inhibiting cancer development and metastasis. We aimed to determine the safe, non-toxic dose of matcha in zebrafish, and to investigate the anticancer effect of matcha on the metastasis and growth of human TBNC cells using a zebrafish xenograft model. Wild-type AB zebrafish were used to conduct multiple general toxicity assessments, including developmental, neuromuscular, and cardiovascular toxicities. 50 µg/ml and 100 µg/ml were determined as safe, non-toxic concentration of matcha in zebrafish. Afterward, the zebrafish xenograft model was successfully established by injection of MDA-MB-468 and MDA-MB-231 human TNBC cells. The tumor size and metastasis of the injected cancer cells were traced through CM-Dil red fluorescent dye. Exposure to non-toxic doses of matcha, showed a trend toward reduction in tumor size of MDA-MB-231 and MDA-MB-468 in a dose-dependent manner. Our results point to a potential dose-dependent anticancer effect of matcha on TNBC tumors; however, more extended observation periods after xenotransplantation are required to confirm the long-term anticancer effect of matcha on tumor growth and metastasis.

Keywords: Anticancer, General Toxicity, Matcha, Zebrafish.

DEDICATION

To my family and loved ones for their endless support.

ACKNOWLEDGMENTS

First, I would like to thank Allah, the God Almighty, for giving me the strength and blessing of completing this thesis. Also, I would like to thank my supervisor Dr. Maha Al-Asmakh and my co-supervisor Dr. Hiba Bawadi very much for their continued guidance, support, and patience. And I extend my heartfelt thanks to Dr. Zain Zakaria for her ongoing assistance and support at each and every step of this research, it wouldn't have been possible without her.

And for sure, I thank my family, especially my mother, for her continuous prayers and for pushing me forward and believing in me more than anyone else. I would also like to thank my dear friends, Alaa Ahmed and Salma Muhammad, for being there for me when it was hard and getting by back, always.

Finally, I would like to express my sincere gratitude to the Biomedical Research Center (BRC) and the zebrafish facility for giving me the opportunity to conduct my thesis research and striving to provide their best support.

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Chapter 1: Introduction

With a high incidence rate, cancer was ranked the second leading cause of mortality globally (Nagai & Kim, 2017). Cancer results from mutations that activate oncogenes or deactivate tumor suppressor genes, which causes uncontrolled cell growth and proliferation and, ultimately, various health issues that might result in death. In Qatar, breast cancer is associated with one of the highest incidence and mortality rates compared to other middle eastern countries (Chouchane, Boussen, & Sastry, 2013), accounting for 14.7% of the total new cancer cases in the country in 2020 (Organization, 2020).

Matcha is the powder form of Japanese green tea (*Camellia sinensis*), cultured under special conditions and processed in a unique way after its collection (Farooq & Sehgal, 2018; Horie, Ema, & Sumikawa, 2017). This process allows matcha to have a high content of caffeine and amino acids, and lower content of catechins compared to other popular types of green tea (GOTO, NAGASHIMA, YOSHIDA, & KISO, 1996; Ikegaya, Takayanagi, & Anan, 1984). However, once dissolved in water, matcha produces 3 times more catechins compared to the loose-leaf form of green tea (Fujioka et al., 2016). Matcha has grabbed the attention of researchers recently, with its multiple potential health benefits, such as enhancing cognitive function and attention (Baba, Kaneko, & Takihara, 2021), improving lipid profile and lowering body inflammation (Zhou, Yu, Ding, Xu, & Wang, 2021), and acting as an anticancer agent (Bonuccelli, Sotgia, & Lisanti, 2018). Studies investigating the anticancer properties of matcha are still limited, where only *in vitro* studies investigate matcha's impact on breast cancer cells, and pointing at a potential anti-proliferative, antioxidant, and cell cycle regulating effect.

Zebrafish animal model is considered a good candidate for investigating the effect of matcha on cancer. That is due to the high resemblance of zebrafish genome to the human genome by around 70% orthologue genes, making zebrafish a useful model in genetic manipulation studies (Howe et al., 2013). Moreover, zebrafish embryos are simple to maintain as they have a short maturation time, fast organ development and can produce hundreds of embryos per mating (Teame et al., 2019). Additionally, the lack of an adaptive immune system of the zebrafish larvae during the first months of development allows the successful xenotransplantation of human cancer cells into the larvae, with little chance of rejection, creating a good model for studying human cancer progression and the efficacy of anticancer compounds (Lam, Chua, Gong, Lam, & Sin, 2004).

Hypothesis

Treating zebrafish xenografted with human breast cancer xenograft by a non-toxic dose of matcha has an anticancer effect compared to non-exposed controls.

Objectives

1. To determine the No Observed Adverse Effect Level (NOAEL) of matcha on the normal development of zebrafish larvae and the neuromuscular and cardiovascular system.
2. To optimize a zebrafish xenograft model of human triple negative breast cancer.
3. To determine the efficacy of matcha as an anticancer treatment through its effect on the zebrafish xenograft model

Chapter 2: Literature Review

Cancer

Cancer is the second leading cause of death globally, just behind cardiovascular diseases (Nagai & Kim, 2017), and its incidence and mortality rates have increased over the years (R. L. Siegel, Miller, & Jemal, 2019). Cancer is defined as unrestrained cell proliferation that acquires metastatic properties in response to oncogene activation and/or tumor suppressor gene inactivation (Sarkar et al., 2013).

Prevalence of Cancer

According to GLOBCAN (2020), the estimated global burden of cancer is 19.3 million new cases, and 10 million deaths in 2020 (Sung et al., 2021). It is predicted that there will be 30 million cancer deaths annually by the year 2030 (Lancet, 2018). In Qatar, the overall cancer incidence rate in 2020 was 1482 cases, with breast cancer becoming the most commonly diagnosed cancer in the Qatari population, accounting for 14.7% of total cancer cases (Organization, 2020).

Breast Cancer

On a global basis, one quarter of all malignancies found in women are breast cancer cases, making it the most commonly diagnosed cancer. According to the American Cancer Society, one in eight women has a lifetime risk of having breast cancer (R. Siegel, 2018). In addition, compared to other Middle Eastern nations, Qatar has one of the highest rates of breast cancer incidence and mortality (Chouchane et al., 2013). Significant progress has been made in the treatment of breast cancer over the past few decades with the identification of intrinsic subtypes of breast cancer, which changed the classification from ductal, inflammatory, or invasive to the expression of molecular markers like the estrogen receptor (ER), progesterone receptor (PR), and the human epidermal growth receptor 2 (HER2) protein. Accordingly, breast cancer is categorized into ER positive (ER+), PR positive (PR+), or HER2 protein positive

(HER2+). 20% of breast cancer cases are HER2+, and about 70% are ER+/PR+ (Perue et al., 2000). Breast cancer can either be negative for all three receptors or positive for two or three receptors. The former scenario is referred to as triple negative breast cancer (TNBC) (Perue et al., 2000). Traditional chemotherapy is the only treatment option for TNBC. Even when patients receive early-stage chemotherapy and endocrine adjuvant treatments, breast cancer still has poor prognosis due to the subsequent occurrence of metastasis in about 30-50% of patients (Lin, 2013; Martin et al., 2017; Roche & Vahdat, 2011). TNBC patients show the poorest prognosis and survival and the highest metastasis prevalence among all breast cancer subtypes (Geyer et al., 2017; Rakha et al., 2007).

Metastasis is known as the spread of cancer cells to different body parts, and it is a very dynamic process (Mansel, Fodstad, & Jiang, 2007). The original tumor does not account for the majority of fatalities in breast cancer patients; instead, spread of cancer cells to other organs does (Weigelt & Peterse, 2005). Stage IV breast cancer is also referred to as metastatic breast cancer, and patients with the disease at this stage have access to the same treatment options as those at other stages. Since no treatments are currently available to prevent metastasis, metastatic breast cancer is regarded as incurable (Roche & Vahdat, 2011).

It can be challenging to assess the effectiveness of anticancer and, more specifically, anti-metastatic drugs in patients with metastatic tumors. Neoadjuvant medicines are being tested in patients with locally advanced cancer to determine if they can effectively eradicate the disseminated cancer cells that cause metastases. Therefore, it is crucial to have reliable and effective cancer experimental models which can recreate settings that allow normal proliferation and migration of cancer cells. *In vivo* models, such as zebrafish, are valuable for gaining insights into the molecular pathways

and clarifying the multicellular connections associated with tumor progression (Vittori, Motaln, & Turnšek, 2015).

Zebrafish as an In Vivo Model

Zebrafish are a relatively recent animal model that is now an important tool for biomedical research. Zebrafish, *Danio rerio*, are small tropical freshwater fish originating from the Ganges River (Tavares & Lopes, 2013). Zebrafish is an attractive animal model due to its high productivity, simplicity in animal care management, suitability for *in vivo* imaging, the similarity of their genome structure to that of humans, and effectiveness of genome editing (Lieschke & Currie, 2007).

Zebrafish are also utilized to assess the carcinogenicity and toxicity of chemicals and medications, and as a model of progression of human cancers (Spitsbergen & Kent, 2003). They respond to carcinogens (Mizgireuv & Revskoy, 2006; Spitsbergen et al., 2000) and mutagens (Beckwith, Moore, Tsao-Wu, Harshbarger, & Cheng, 2000) spontaneously, which leads to the development of malignant tumors (Beckwith et al., 2000). Additionally, transgenesis of mammalian cancer genes has been demonstrated to establish numerous major human tumor forms zebrafish, proving that the molecular processes behind mammalian cancer are also present in zebrafish (Lieschke & Currie, 2007). Importantly, previous studies have demonstrated that human cancer cells, including breast cancer cells can grow, proliferate, metastasize, and trigger angiogenesis in zebrafish, similar to mouse xenograft models (Marques et al., 2009).

Zebrafish offer an alternate platform which can replace murine animal models of cancer (Yen, White, & Stemple, 2014). The usual use of mouse models for testing anticancer drugs has some limitations that restrict their use for studying metastasis.

Early stage metastasis is challenging to evaluate, the mouse metastatic process takes a long time, and animal euthanization is necessary to analyze malignant lesions (Zhao, Huang, & Ye, 2015). Zebrafish can be used to study certain phases of metastasis, albeit it can be challenging to model the complete process. Human cancer cells, including breast cancer, were able to multiply, spread, and induce angiogenesis in zebrafish, similar to mouse xenograft models (Marques et al., 2009). Proto-oncogenes, angiogenic factors, tumor suppressors, cell cycle, and extracellular matrix proteins in the human genome were found to be all highly conserved in both sequence and function in zebrafish (Zon & Peterson, 2005). Due to the fact that zebrafish do not have an adaptive immune system that is active and completely functional until about day 28 post fertilization, this enables the grafting of human cells without fear of rejection (Lam et al., 2004). Because they are transparent, transgenic zebrafish allow the investigation of tumor spread *in vivo*. Additionally, the fish are simple to handle, can be kept in small amounts of water, may be placed in multi-well cell culture plates, and offer a noninvasive cancer model to examine the effects of exposure on the advancement of cancer using high resolution microscopy (Parng, Seng, Semino, & McGrath, 2002; Wehmas, Tanguay, Punnoose, & Greenwood, 2016; Zampedri, Martínez-Flores, & Melendez-Zajgla, 2021).

Matcha, a Natural Product, as an Anticancer Compound

Natural products are desirable prospects for developing anticancer drugs due to their diversity in chemical composition and biological activities. Japanese green tea (*Camellia sinensis*) leaves are commercially available in a powder form called matcha. Matcha differs from other common green tea varieties in that it is produced, collected, and processed in a special way, resulting in a tea product high in theanine and caffeine but lower in catechins. Research has shown that matcha has multiple health advantages,

such as an improvement in cognitive function, cardiometabolic health, and anti-tumor activity (Sokary, Al-Asmakh, Zakaria, & Bawadi, 2023). Natural antioxidants like polyphenols, which comprise as much as 30% of matcha's dry weight (Komes, Horžic´, Belšc˘ak, Ganic´, & Vulic´, 2010; Mandel et al., 2005), have long been associated with the health advantages of Japanese green tea (Kurleto et al., 2013). Similar to other powerful antioxidants such as vitamins C, E, carotene, and tocopherol, polyphenols are also strong antioxidants. Catechins account for 90% of these polyphenols in matcha (Tachibana, 2009). Of these catechins, epigallocatechin-3-gallate (EGCG) is the most prevalent and active catechin, and is also abundant in matcha (Kochman, Jakubczyk, Antoniewicz, Mruk, & Janda, 2020). Numerous studies indicated that EGCG could be a potential anticancer agent. According to a recent meta-analysis of *in vitro* studies, EGCG was found to be efficient in reversing estrogen receptor gene expression, inducing epigenetic modifications and events, and inhibiting tumor growth rate (effect size = 2.84, 95% CI: 2.60-3.10) (Gianfredi et al., 2017). Accordingly, this points to the potential of matcha to produce a similar anti-tumor effect, especially since matcha produces 3 times more catechins once it is dissolved in hot water compared to other popular teas, including leaf-form green tea (Fujioka et al., 2016). Another bioactive compound available in matcha with potential anticancer effects is theanine. Theanine is a type of amino acid that is not derived from protein and is found mainly in tea, especially matcha. A recent study (Shojaei-Zarghani, Rafraf, & Yari-Khosroushahi, 2021) has systematically examined the available evidence on the anticarcinogenic and anticancer effects of theanine from natural sources. According to the evidence from 14 studies conducted *in vitro*, *ex vivo*, and *in vivo*, theanine has a moderate inhibitory effect on the migration, apoptosis, invasion, metastasis, and proliferation of cells in several types of cancer. Animal experiments have demonstrated that theanine can exert its

anticancer effect by inhibiting several signaling pathways while also activating programmed cell death in a caspase-independent manner (Shojaei-Zarghani et al., 2021). However, research regarding matcha's ability to fight tumors is in its early stages, with only 3 *in vitro* studies conducted to date to investigate matcha's impact on breast cancer cells (Table 1). The findings from these studies demonstrate that matcha can considerably impact survival, proliferation, antioxidant response, and cell cycle regulation of breast cancer cells (Bonuccelli et al., 2018; Keckstein et al., 2022; Schroder et al., 2019). Although evidence from *in vitro* studies indicates a protective effect against triple negative breast cancer cells, an *in vivo* animal model is needed to confirm this effect in a more robust and systematic evaluation of the multicellular connections associated with tumor progression. Since the anticancer effects of matcha have never been investigated *in vivo* in any animal cancer models, our study will be the first to investigate this outcome.

Several studies have explored the impact of individual active compounds found in green tea on various types of cancer using zebrafish as an *in vivo* model. One compound, Theabrownin, which is present in different teas and possesses antioxidant and anticancer properties, has been observed to cause DNA damage and trigger programmed cell death (apoptosis) in osteosarcoma cells transplanted into zebrafish larvae (specifically the U2OS cell line). This effect relies on a mechanism involving p53 protein (Jin et al., 2018). Furthermore, Theabrownin has demonstrated significant inhibition of the growth of two types of non-small cell lung cancer in zebrafish larvae that received xenograft transplants of H1299 and A549 cell lines (X. Xiao et al., 2022). It also suppressed migration and induced apoptosis of cancer cells. Additionally, Theabrownin has exhibited remarkable suppression of hepatocellular carcinoma growth in a zebrafish model that received xenograft transplants of the SK-Hep-1 cell

line (Jiaan Xu et al., 2022). Another active compound and polyphenol in tea, Theaflavin, caused significant inhibition of A375 tumor growth in larval zebrafish, which represents a melanoma cell line (L. Zhang et al., 2020).

Table 1. Summary of the studies assessing the anticancer effect of matcha.

Reference	Type	Year	Dose	Methods	Results
(Keckstein et al., 2022)	*In vitro* (T47D, triple negative breast cancer cells)	2022	5, 10, and 50 µg/ml matcha	Cell viability (WST-1 proliferation assay, measured as optical density) PPARγ expression (PCR and Western Blot)	⬇ cell viability ⬆ PPARγ expression
(Schroder et al., 2019)	*In vitro* (MCF7 and MDA-MB-231 triple negative breast cancer cells)	2019	Green tea or Matcha in Ethanol or water: 3000 µg/ml vs EGCG vs quercetin and tamoxifen	MTT test for viability BrdU assay ATP luminescence test/ CellTiter-Glo® test Neutral Red test for viability Oxidative stress by measuring H_2O_2 concentration	⬇ Viability and Proliferation
(Bonuccelli et al., 2018)	*In vitro* (MCF7, triple negative breast cancer cells)	2018	Control vs doxycycline vs 0.2 mg/ml matcha	Ingenuity Pathway Analysis (IPA) Oxygen consumption rate (OCR) Extracellular acidification rate (ECAR) Quantitative proteomic analysis	⬇ Mitochondrial metabolism ⬇ Glycolysis and glycolytic capacity ⬇ Cell viability ⬇ Sphere-forming ability ⬇ Oxygen consumption rate and ATP production

Chapter 3: Materials and Methods

Methods

Matcha Extract Preparation

Ceremonial grade organic matcha was purchased from Wilderness Poets, USA, and stored in a refrigerator at 4 ˚C. The extract was prepared, according to previous literature (Li et al., 2019; Q. Zhang et al., 2022), by boiling 50 mg of matcha in 50 ml Embryo medium (E3M) at 150˚C on a hot plate for 20 min, with continuous stirring using a magnetic stirrer. A calibrated sensitive scale was used to weigh the matcha powder, and a 10 ml pipette from Eppendorf was used to obtain an accurate volume of E3M. Before placing the flask on the hot plate, it was covered using foil to prevent water loss through vapor. The matcha extract solution was then allowed to cool down first. The extract was centrifuged at 2500 rpm for 3 minutes, and the supernatant was isolated and used as the stock solution with a concentration of 1mg/ml. The dilutions were prepared under the hood for sterility. For each experiment, the extract was freshly prepared.

Animal Care and Handling

Animal experiments were all conducted in accordance with the national and international guidelines for the use of zebrafish in experimental settings (Reed & Jennings, 2011). All experiments followed the animal protocol guidelines required by Qatar University and the Policy on Zebrafish Research established by the Department of Research in the Ministry of Public Health, Qatar. The experimental design for this study was approved by the Institutional Biosafety Committee (IBC) at Qatar University, with the approval document (QU-IBC-2022/014) attached in the appendix, chapter 6.

Zebrafish Maintenance and Breeding

Zebrafish are housed in recirculating stand-alone aquarium racks, where the water is kept at a temperature of ~ 28°C, and the room is kept at 26°C. The room has a 14-hour diurnal light schedule (from 7:30 am to 9:30 pm) with 10 hours of darkness (9:30 pm to 7:30 am). Zebrafish are raised and maintained in normal laboratory settings as outlined by Westerfield (Westerfield, 2000) to ensure high quality embryos. Male and female zebrafish were kept apart in a mating basket the night before mating; the next morning, as the lights were turned on, the barrier was removed, and the zebrafish were left to mate in shallow water undisturbed for about 20 minutes. The eggs were then collected and cleaned in E3M, which has also been prepared according to Westerfield (Westerfield, 2000). E3M contains NaCl, KCl, $MgSO_4$, $CaCl_2$ and methylene blue in water purified by reversed osmosis. The fertilized and alive eggs were placed in a petri dish with E3M. The embryos were divided into two to three duplicates for each group: control (E3M), positive control (20 ug/ml Zinc Oxide), and the different experimental matcha concentrations. The initially chosen concentrations were based on multiple previous optimization experiments designed to identify the range of the non-lethal concentration of matcha. The embryos were then incubated at a temperature of 28 °C. Up to the time of each experiment, dead embryos were removed daily with minimal removal of E3M. Only wild-type AB zebrafish embryos aged less than 5 days were used for all of the conducted experiments.

Determination of No Observed Adverse Effect Level (NOAEL) in Zebrafish Larvae

The no observed adverse effect level (NOAEL) is the highest matcha concentration at which larvae exposed for 96 hours show no signs of abnormalities (such as curved body axis, yolk sac, and pericardial edema). In a 6-well cell culture plate, each experimental group consisted of 10 larvae and matching negative and

positive controls that were given E3M and ZnO (20 µg/ml), respectively. The larvae were examined under a standard microscope every 24 hours for survival and any apparent phenotypic defects. After concluding all toxicity assessments, a matcha concentration was chosen based on the NOAEL, which is the concentration that did not result in any obvious developmental abnormalities nor toxicity in the zebrafish. Zinc Oxide at 20 µg/ml concentration was used as the positive control due to its known effects of delaying zebrafish embryo and larva development, causing lower survival and hatching rates, and inducing tissue damage (Zhu et al., 2008).

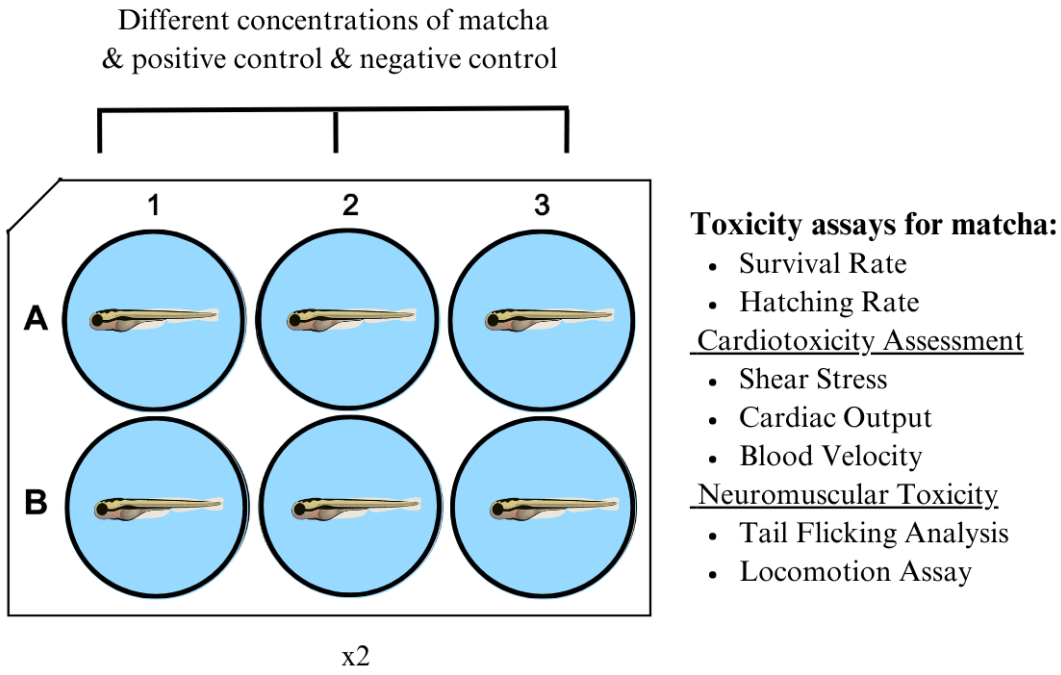


Figure 1. Toxicity Assessment of matcha in vivo to determine the No Observed Adverse Effect Level (NOAEL).

Developmental Toxicity: Survival Rate and Hatching Rate Analyses

The toxic effect of matcha was determined for each matcha treatment group (*i.e.*, different matcha concentrations) using standard assays, including survival rate and

hatching rate. Each group's dead, surviving, and deformed embryos were monitored and reported every 24 hours post fertilization and until the end of the experiments using a standard examining microscope. Survival rate was calculated as the number of alive embryos out of the total number of incubated embryos multiplied by 100. After every 24 hours, the dead embryos were removed to prevent infections or influencing the survival of the alive embryos. Additionally, starting at 48 hpf, the hatching rate was assessed as the percentage of hatched embryos out of the overall number of incubated embryos for each group.

Cardiotoxicity Assessment: Live Imaging of Zebrafish

The anatomy of the zebrafish heart is composed of one atrium and one ventricle; the major blood vessels they connect to are the Dorsal Aorta (DA) and Posterior Cardinal Vein (PCV) (Shin, Pomerantsev, Mably, & MacRae, 2010; H. C. Yalcin, Amindari, Butcher, Althani, & Yacoub, 2017). Several hemodynamic parameters were assessed through live imaging and tracking of the red blood cells movement in the DA from the trunk of the larvae. These parameters include cardiac output, blood flow, velocity, vessel diameter, and heartbeat. This was conducted at 72 hpf by stabilizing zebrafish larvae from each treated and control group using 3% methylcellulose on a depression slide with concave wells. Larvae were then positioned correctly to be able to visualize the main vessels in the trunk area. After the larvae were stable, high-speed time-lapse videos were recorded utilizing a Zeiss Lumar V12 stereo microscope equipped with a Hamamatsu Orca high-speed camera and HCImage software. Bright field videos were recorded for 10 seconds for each embryo at 100 frames per second and 100 × magnification. MicroZebraLab blood flow software from Viewpoint (version 3.4.4, Lyon, France) was utilized to analyze the recorded videos and estimate the blood velocity, vessel diameter, and heartbeat by tracking red blood cells. The

fractional shear stress levels were estimated using blood velocity measurements according to Benslimane et al. (Benslimane et al., 2020). The formula ($\tau = (4\ \mu V_{mean})/D$) calculates shear stress (τ, dynes/cm^2), where μ is the blood viscosity (dynes/cm^2), V is the average blood velocity (µm/s), and D is the vessel diameter (µm). To calculate the cardiac output (nL/min), the formula ($F = V \cdot A$) was used, where V is the average blood velocity (µm/s), and D is the vessel diameter (µm). Many published articles explained zebrafish's heart function analysis technique (H. Yalcin, Abuhabib, Kitaz, Mohamed, & Zakaria, 2018; H. C. Yalcin et al., 2017).

Neuromuscular Toxicity: Behavioral and Locomotion Assay

To investigate the neuromuscular toxicity of matcha on zebrafish embryos, zebrafish larvae at 96 hpf were individually separated in E3M in a 96-well flat-bottom cell culture plate. The embryos were left in an incubator for one hour to acclimatize to the new environment. Next, the plate was placed in the Viewpoint ZebraLab chamber (Noldus Information Technology, Wageningen, The Netherlands) to automatically track the Zebrafish embryos' movement. The chamber was at 28˚C and was illuminated with white light. The assessment started after a 20-minute acclimatization period in the dark, then alternating 10 minutes of light, and dark were scheduled for 60 minutes. Calculations of the larvae movement were recorded every 5 minutes until the end of the experiment. Detection and field settings were adjusted to achieve optimal tracking. (Summarized in Figure 2)

Xenograft Zebrafish Model

Cell Culture

American Type Culture Collection (ATCC) (Rockville, MD, USA) guidelines were followed for cultivating and maintaining MDA-MB-468 and MDA-MB-231 cell lines (ER^-, PR^-, $HER2^-$). MDA-MB-468 and MDA-MB-231cells were cultured in

RPMI-1640 and DMEM (ThermoFisher Scientific), respectively. Both media were supplemented with 10% fetal bovine serum (ThermoFisher Scientific) and 1% penicillin/streptomycin antibiotics (Sigma-Aldrich, MO) and 1% L-Glutamine (ThermoFisher Scientific). Cells were incubated at 37 °C with 5% CO_2 and 76% humidity and growth media was changed every alternate day to provide the optimum growth environment for cells to continue growing exponentially until they are ready for microinjection. Cells were confirmed to be confluent under a standard microscope before being used in xenograft experiments.

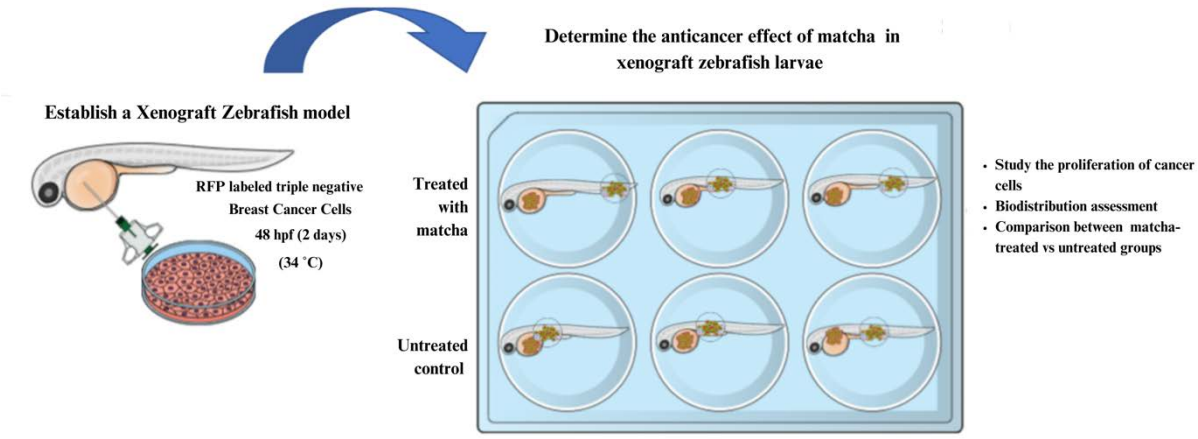


Figure 2. Protocol for the establishment of a xenograft zebrafish model.

Fluorescent Labeling of Breast Cancer Cells Prior To Xenotransplantation

The staining of cells with the fluorescent dye were optimized first. MDA-MB-468 and MDA-MB-231 cells are adherent cells cultured on a sterile glass coverslip placed in a small petri dish and maintained in growth media for 24 hours. The staining solution was prepared using CM-Dil fluorescent dye (V22888, Invitrogen) which was freshly prepared for each experiment by adding 1µL of the dye labeling solution to 200 µL of growth media. Once the breast cancer cells were confluent (more than one million

cell/ml), growth media was drained off the coverslip. Afterwards, 100 µl of the staining medium was placed onto the corner and gently shaken to distribute it over the entire coverslip and put back into the incubator. After incubation, the staining solution was removed, and the coverslip was washed with warmed growth media and incubated for 10 minutes thrice. The cells were then checked under an EVOS M5000 fluorescence microscope with an RFP filter applied in a dark room to confirm uniform staining and adequate fluorescence. Several trials were conducted to determine the ideal incubation time for the two cell lines, and the optimum fluorescence and staining results were determined the optimal incubation period to be 20 minutes of incubation with the staining solution.

For xenotransplantation into zebrafish, cells were harvested by trypsinization and pelleted by centrifugation at 1500 rpm for 5 minutes. The supernatant was discarded, and the cells were re-suspended in serum-free growth media. The cell suspension was then ready to be used in the microinjection procedure. Cells were promptly stained with CM-DiI for 20 minutes, and prepared for each injection trial, which was carried out within 2 hours. The number of cells contained per milliliter was counted using Countess 3 Automated Cell Counter (ThermoFisher Scientific) by mixing 10 µl of the cell suspension with 10 µl of trypan blue dye, then loading 10 µl of the mixture to a Countess Cell Counting Chamber Slide (ThermoFisher Scientific). The machine generates the number of alive cancer cells and the percentage of fluorescent cells. Experiments were conducted when more than 80% of the cancer cells emit fluorescence.

Preparation of Zebrafish for Microinjection

Two days before the microinjection, the adult zebrafish were allowed to breed. The following morning, eggs were retrieved, dead embryos were removed, and the

remaining embryos were subsequently incubated at 28˚C. At 2 days post fertilization, the chorion was removed by exposing the larvae to Pronase at 1 mg/ml from a stock of 10 mg/ml, then incubating it for 5 minutes at 28˚C. Finally, the chorions of any yet unhatched embryos were removed by gentle pipetting using a transfer pipette. Afterwards, larvae were washed with fresh E3M three times and incubated at 28˚C until required for injection. At the time of injection, the larvae were transferred to the injection mold and anesthetized by exposing them to 0.003% tricaine methane sulfonate for 2 minutes, prepared from a stock solution of 4 mg/L concentration, 9 µl of stock was added to 30 ml of E3M. Afterwards, zebrafish larvae were placed in the dorsal position in a petri dish coated with agarose gel and molded into furrows (Figures 3-A and 3-B). As for the needles used for microinjection, Wehmas' recommendation (Wehmas et al., 2016) was followed, and the borosilicate capillaries were pulled with the Sutter Instrument P-20 at the following settings: pull = 20; velocity = 50; time = 200; air pressure = 200; and heat = ramp +21°C.

Xenotransplantation of Fluorescently Labeled Human TNBC Cells In Zebrafish Larvae

MDA-MB-468 and MDA-MB-231 human TNBC cells labeled with red fluorescent dye were xenotransplant into 2-days-old zebrafish larvae. After being redissolved in 200 µl of serum-free DMEM or RPMI-1640, the cancer cell suspension was loaded into the drawn needle fitted with Eppendorf capillary tips. Each needle contained five microliters of the tagged cell suspension. Next, the needle was put into the micromanipulator's orifice. The microneedle was angled at a 45-degree angle (Figure 3-C). After the drawn needle's tip was cut using forceps, 1-2 nL of labeled breast cancer cells were injected into the zebrafish yolk for a total of about n = 30 larvae per experimental group. Also, 30 larvae were kept un-injected (cancer-free) to serve as the negative control. After the injection, the larvae were allowed to recover for half an

hour. Four exposure groups were created: negative control (cancer-free larvae in E3M), control (larvae injected with cells and treated with E3M), injected larvae without matcha treatment, and two matcha concentrations (larvae injected with cells and exposed to 50 µg/ml and 100 µg/ml matcha). Larvae were placed in 6-well cell culture plates (with 20–30 larvae per well). Although roughly 30 larvae were injected for each treatment group, death, edemas, or having no cancer cells at 1 day post injection (dpi) caused some larvae to be eliminated from further evaluation.

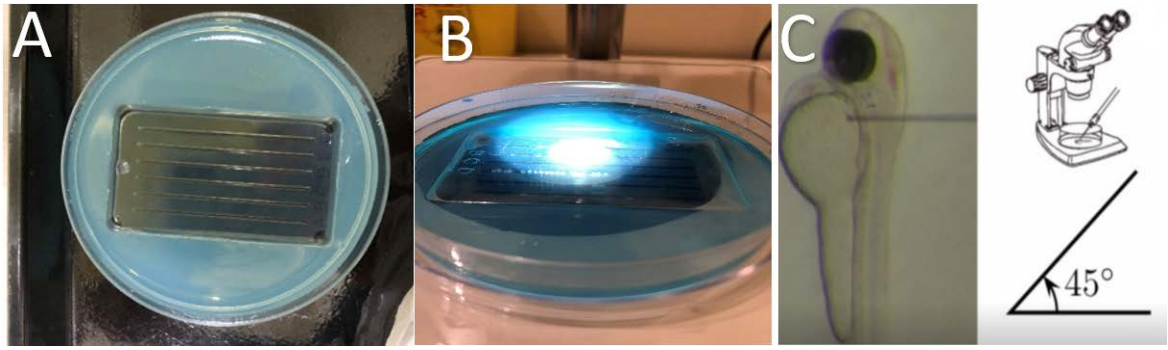


Figure 3. Steps for injection of cancer cells into the yolk of 48 hpf (2 days) zebrafish larvae. (A) Injection plate; (B) The embryos in the injection furrows; (C) Angle for microinjection.

Imaging Breast Cancer Cell Progression in Zebrafish Larvae

At one dpi, the xenotransplant zebrafish larvae were placed on a concave glass slide after being anesthetized in a 0.003% tricaine methane sulfonate solution. To examine the yolk sac, the larvae were placed using a pipette tip in a lateral position to detect the fish with cancer cells and eliminate the ones that do not have cancer cells or are deformed. Imaging was carried out using an EVOS M5000 fluorescence microscope with an RFP filter in a dark room. To assess the proliferation and metastasis of cancer cells as well as the impact of matcha on the quantity of cancer cells, larvae were

photographed comparably at 1 dpi and 2 dpi and compared to the injected control not exposed to matcha. Images were captured at 4X magnification, and a 10X magnification picture was captured for the yolk sac, the injection region.

Quantification of Breast Cancer Cell Fluorescence

To determine the number of cancer cells in the developed xenograft model and demonstrate matcha's impact on tumor mass size and metastasis, captured images were used to quantify the fluorescent signals emitted by the RFP labelled TNBC cells. Image J software (National Institutes of Health, Bethesda, MD) was used, and the readings were corrected to eliminate background fluorescence and obtain the corrected total cell fluorescence (CTCF).

Statistical Analysis

Statistical analysis was performed using GraphPad Prism version 9.51 software. Data were analyzed using one way-ANOVA with Dunnet's multiple comparison test. Statistical significance was considered when the p-value was less than 0.05. One asterisk (*) indicates $p<0.05$, two asterisks (**) indicate $p<0.01$, three asterisks (***) indicate $p<0.001$ and four asterisks (****) indicate $p<0.0001$.

Table 2. List of materials.

Material	Company
Ceremonial Grade Organic Matcha Tea	Wilderness Poets, USA
MDA-MB-468 cell line	ATCC, Manassas, VA, USA)
MDA-MB-231 cell line	ATCC, Manassas, VA, USA)
RPMI Medium 1640 (1X)	Gibco® Laboratories, Thermo Fisher Scientific, USA)
DMEM Medium	Gibco® Laboratories, Thermo Fisher Scientific, USA)
GlutaMAX	Gibco® Laboratories, Thermo Fisher Scientific, USA
Fetal Bovine Serum	Gibco® Laboratories, Thermo Fisher Scientific, USA
Penicillin-Streptomycin Antibiotic	Gibco® Laboratories, Thermo Fisher Scientific, USA

DPBS (1X)	Gibco® Laboratories, Thermo Fisher Scientific, USA
Trypsin	Gibco® Laboratories, Thermo Fisher Scientific, USA
Zinc Oxide	Thermo Fisher Scientific, USA
Vybrant™ CM-DiI Cell-Labeling Solution	ThermoFisher Scientific, USA
Countess Cell Counting Chamber Slide	ThermoFisher Scientific, USA
Tricaine methane sulfonate 98%	Sigma Aldrich, Germany
Methylcellulose	Sigma Aldrich, Germany
Wild-type AB zebrafish embryos	
Embryo media (E3M)	
Agarose Gel	

Table 3. List of equipment.

Equipment	Company
EVOS M5000 fluorescence microscope	Thermo Fisher Scientific, USA
Viewpoint ZebraLab chamber	Noldus Information Technology, Wageningen, The Netherlands
Countess 3 Automated Cell Counter	ThermoFisher Scientific, USA
Concave microscope slide	
Confocal Microscopy	
Autoclaved Glass Coverslips	
100 ml Capacity Glass Beaker	
60 mm Petri Dishes	
6-well cell culture plates	
96-well flat-bottom cell culture plate	
Pipettes and micropipettes	
Magnetic Stirrer rod	
Hot plate	
Sensitive Balance	
Centrifuge	
Borosilicate capillaries	
Zebrafish Incubator (28˚C / 34˚C)	
Cell Culture Incubator (37 °C, 5% CO_2 and 76% humidity)	
Zeiss Lumar V12 stereo microscope	

Chapter 4: Results

Developmental Toxicity of Matcha in Zebrafish Larvae

Survival Analysis

To determine the safe dose of matcha on zebrafish larvae, multiple concentrations were tested, and the survival rate was calculated every 24 hours post fertilization. Figure 4 shows the survival rate for the various experimental groups after 24 hours of fertilization. Matcha concentrations at 200 and 250 µg/ml significantly decreased the survival of the zebrafish embryos ($p < 0.05$). Higher concentrations (300 µg/ml and higher) caused a more profound death rate ($p < 0.0001$). Overall, there was a dose-dependent effect on the survival of the embryos where higher concentrations of matcha decreased survival at 24 hours post fertilization. However, the lower concentration of matcha (≤ 150 µg/ml) did not significantly affect the survival of the zebrafish larvae. As expected, the positive control (20 µg/ml Zinc Oxide) did not significantly affect the survival rate. The lethal concentration 50% (LC50) of matcha was found to be at 149.8 µg/ml, which is the concentration at which 50% of the embryos die due to matcha exposure.

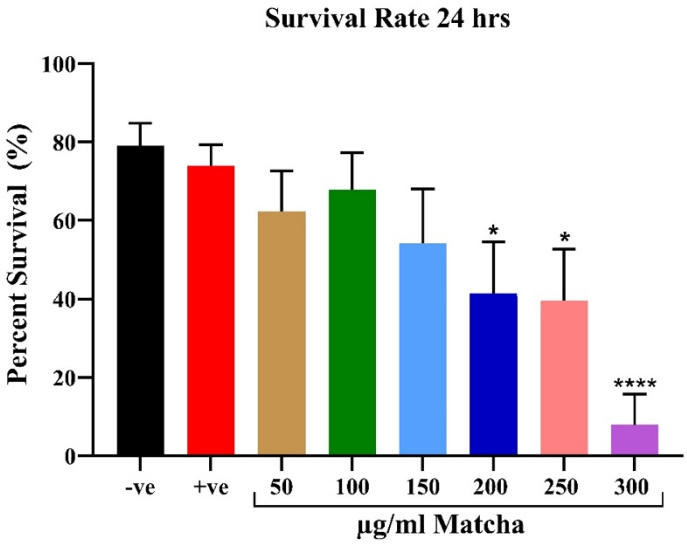


Figure 4. Survival rate at 24 hours post fertilization for various concentrations of matcha. Embryos were visualized and counted using a standard examining microscope. The survival rate was calculated as the number of surviving embryos divided by the total number of embryos used. Data are represented as percent survival (n=30 embryos per group; the experiment was performed in triplicate). Analysis was done by one-way ANOVA with Šídák's multiple comparisons test. *p<0.05, and ****p<0.0001. Abbreviations: -ve, negative control; +ve, positive control.

Hatching Rate Analysis

The hatching rate represents the number of embryos that hatch from their chorions divided by the total number of surviving embryos. Zebrafish larvae normally start hatching after 48-52 hours post fertilization. Based on the survival results above, the maximum concentration of matcha used to assess the hatching rate was 250 µg/ml. Shown in Figure 5 is the effect of matcha on zebrafish embryos' hatching rate, which is usually completed by 72 hours post fertilization. A dose-dependent decrease in hatching is seen at higher concentrations of matcha, especially at 150 µg/ml concentration and higher. The lower concentrations (100 µg/ml and lower) caused a non-significant

decrease in the hatching rate compared to the negative control. The positive control affected the hatching rate by causing 0 % hatching of the embryos at all time points, which is the expected effect of ZnO on zebrafish hatching.

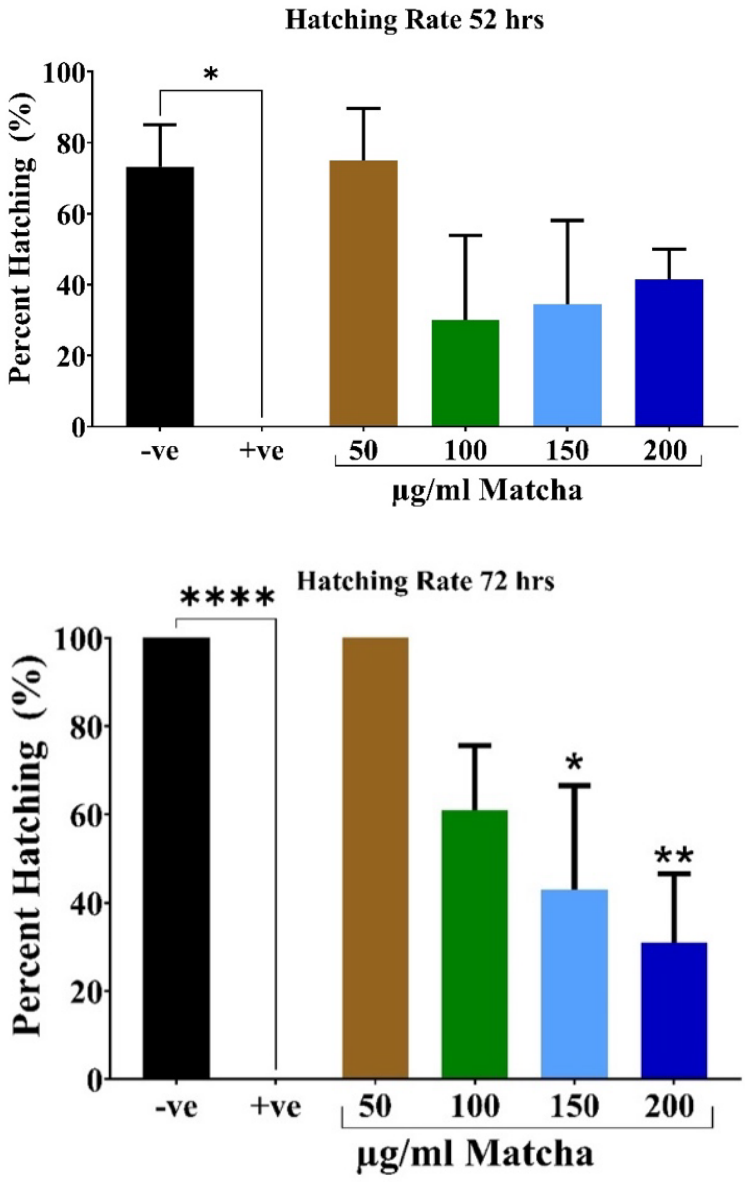
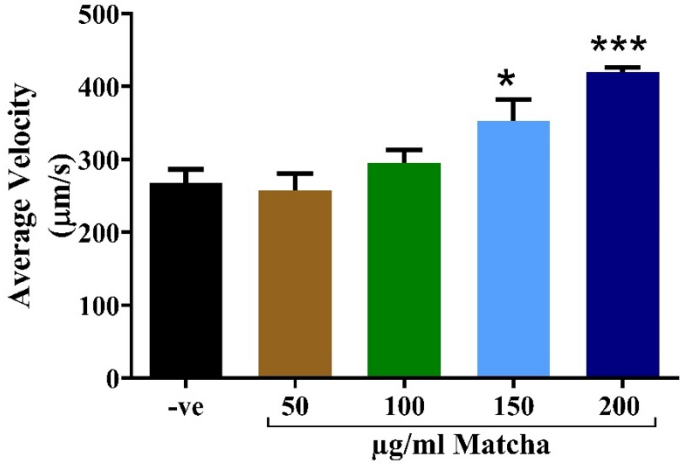


Figure 5. The effect of matcha on the hatching of zebrafish embryos at 52- and 72-hours post fertilization. Embryos were visualized and counted using a standard examining microscope. The hatching rate was calculated as the number of hatched embryos divided by the total number of surviving embryos. Data are represented as percent hatching (%) (n=30 embryos per group; the experiment was performed in

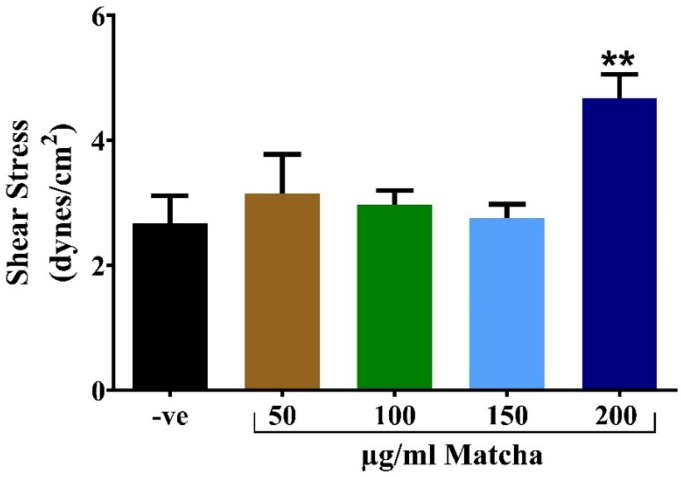
triplicate). Analysis was done by one-way ANOVA with Šídák's multiple comparisons test. *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001. Abbreviations: -ve, negative control; +ve, positive control.

Cardiotoxicity Assessment

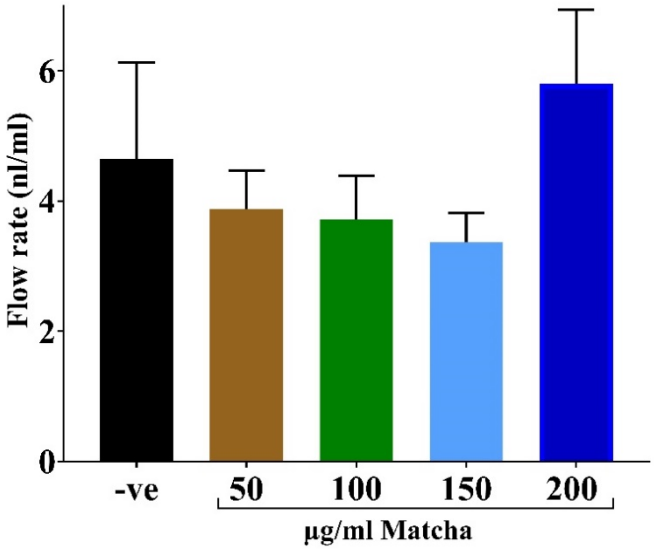
Following hatching rate analysis, the cardiac function parameters were assessed on one of the zebrafish's main vessels, which is the Dorsal Aorta (DA), a main vessel in zebrafish, at 72 hours post fertilization. As shown in Figure 6-A, the Aorta blood flow velocity was significantly increased by exposure to 150 µg/ml of matcha ($p < 0.05$), and a more substantial increase was seen with exposure to 200 µg/ml of matcha ($p < 0.001$). Additionally, the Aorta shear stress was significantly increased by exposure to 200 µg/ml matcha concentration ($p < 0.01$; Figure 6-B). However, the cardiac output (Figure 6-C) was not significantly affected by exposure to matcha, although 200 µg/ml of matcha caused a trend towards increased cardiac output compared to the other concentrations, but without statistical significance ($p > 0.05$). No significant difference was detected in the vessel diameter analysis (Figure 6-D) and the heart pulse (Figure 6-E), showing no specific trend across all matcha concentrations.



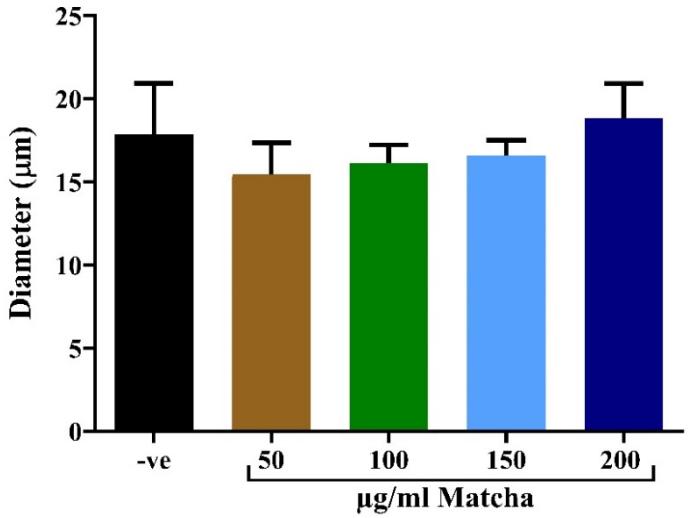
(A)


Shear Stress (dynes/cm^2)
6
4
2
0
**
-ve
50
100
150
200
µg/ml Matcha

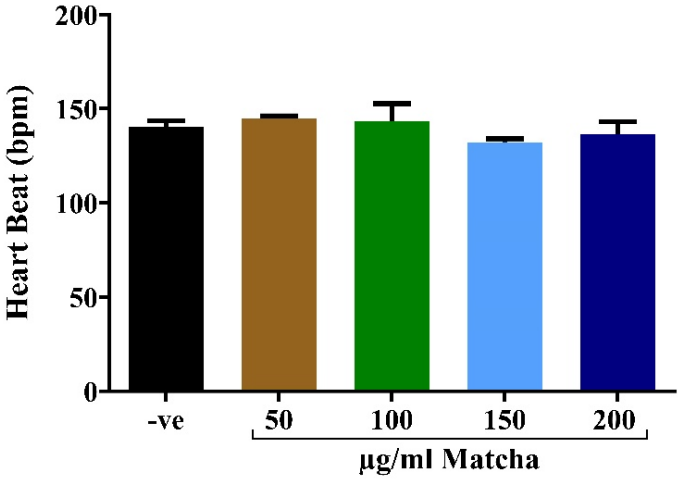
(B)


Flow rate (nl/ml)
6
4
2
0
-ve
50
100
150
200
µg/ml Matcha

(C)



(D)



(E)

Figure 6. Assessment of the cardiac parameters measured at the dorsal aorta at 72 hours post fertilization. Embryos were stabilized in 3% methylcellulose and visualized using Zeiss SteREO discovery V8 Microscope equipped with Hamamatsu Orca Flash high-speed camera. A workstation computer and HCImage software were used for video acquisition. A 10-second video at 100X magnification of the trunk region was recorded for each embryo. Videos were analyzed to detect the dorsal aorta blood flow velocity (µm/s) (A), shear stress (dynes/cm^2) (B), flow rate (nL/min) (C), vessel diameter (µm) (D), and arterial pulse (bpm) (E). All data are represented as mean ±

SEM (n=5 for all groups). Analysis was by one-way-ANOVA with Šídák's multiple comparisons test. *p<0.05, **p<0.01, and ***p<0.001. Abbreviations: -ve, negative control; BMP, beats per minute.

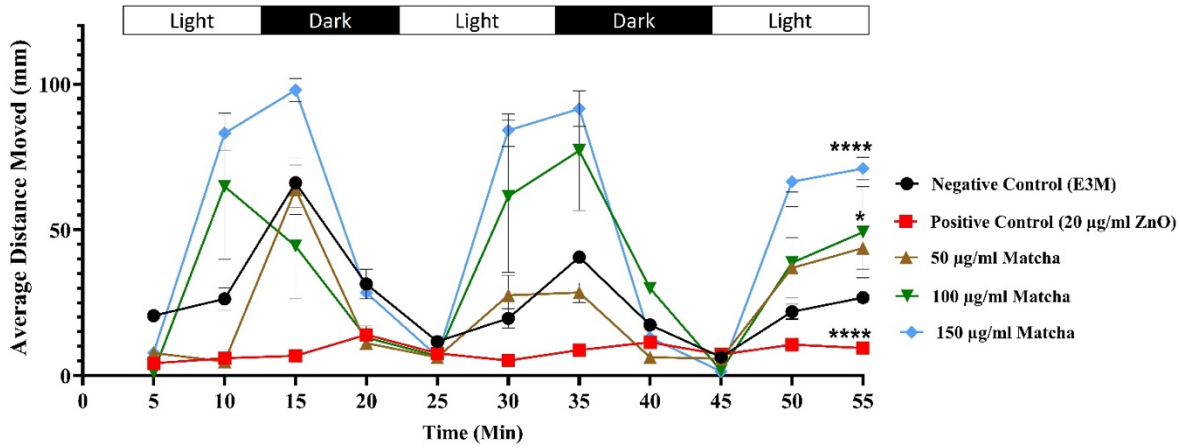
According to the results of the cardiotoxicity analysis, matcha concentration 200 µg/ml was not considered for further analysis due to its detrimental effect on blood flow velocity and shear stress. Therefore, the concentrations 50, 100 and 150 µg/ml were used further to assess the neuromuscular toxicity of matcha on zebrafish larvae.

Neuromuscular Toxicity: Behavioral and Locomotion Assay

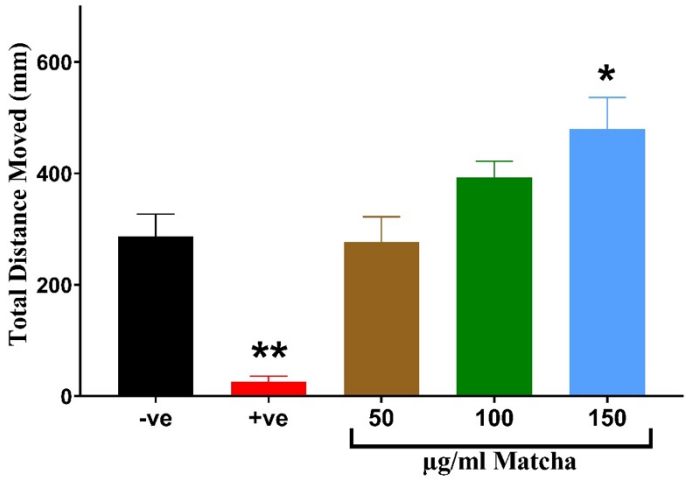
Locomotion assay of the treated versus control groups was conducted to investigate the effect of matcha on the neuromuscular system of zebrafish larvae. Locomotion assay was conducted at 96-hours post fertilization, measuring the average and total distance moved by each larva in millimeters. After an initial 20 minutes acclimatization period, the measurements were set to be calculated every 5 minutes during 60 minutes of alternating 10 minutes light/dark cycles as shown in Figure 7-A; matcha concentrations higher than 50 µg/ml significantly increased the distance moved by the larvae, which are at 100 and 150 µg/ml of matcha. The effect of 100 µg/ml of matcha significantly increased the average distance moved by the larvae ($p < 0.05$).

In comparison, 150 µg/ml caused a higher increase in the average moved distance and more substantial statistical significance ($p < 0.0001$). Moreover, the positive control significantly reduced the movement of the larvae when compared to the negative control ($p < 0.0001$). Overall, the results showed a regular motion pattern where larvae movement increased in light and decreased in the dark. This pattern was seen for both treated and control zebrafish groups.

Figure 7-B shows the total distance moved by the larvae during the locomotion assay. Compared to the negative control, the positive control significantly reduced the total motion of the zebrafish larvae ($p < 0.01$). At the same time, 150 µg/ml of matcha significantly increased the total distance moved by the larvae exposed ($p < 0.05$).



(A)



(B)

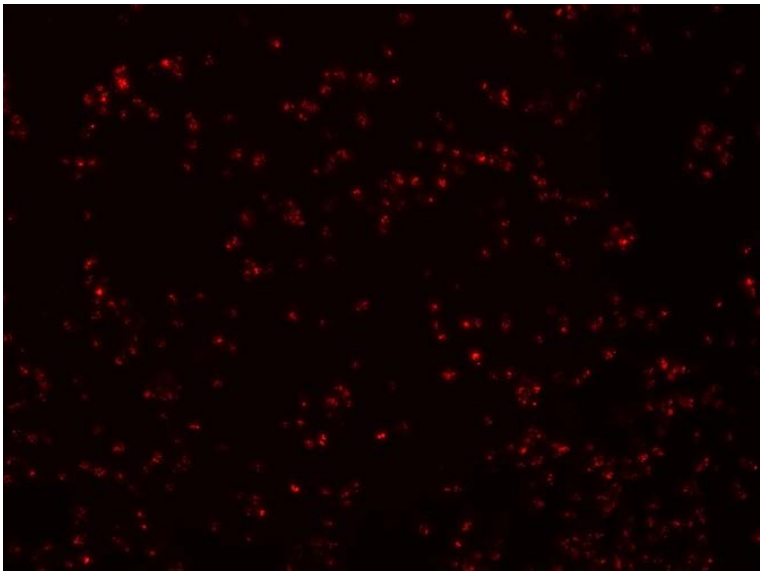
Figure 7. Behavioral and locomotion assay of the experimental groups at 96 hours post fertilization. Single embryos were placed in the Viewpoint ZebraLab chamber and exposed to alternating 10 minutes light/dark cycles for 60 minutes. The movement of the embryos was tracked to measure the distance moved by each larva in millimeters. (A) The average distance moved by all embryos in each experimental group was

measured every 5 minutes of the assay. (B) The total distance moved was measured for all embryos in the exposure group by the end of the assay. All data are represented as mean ± SEM. Analysis was done by one-way-ANOVA with Šídák's multiple comparisons test. *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001. Abbreviations: -ve, negative control; +ve, positive control.

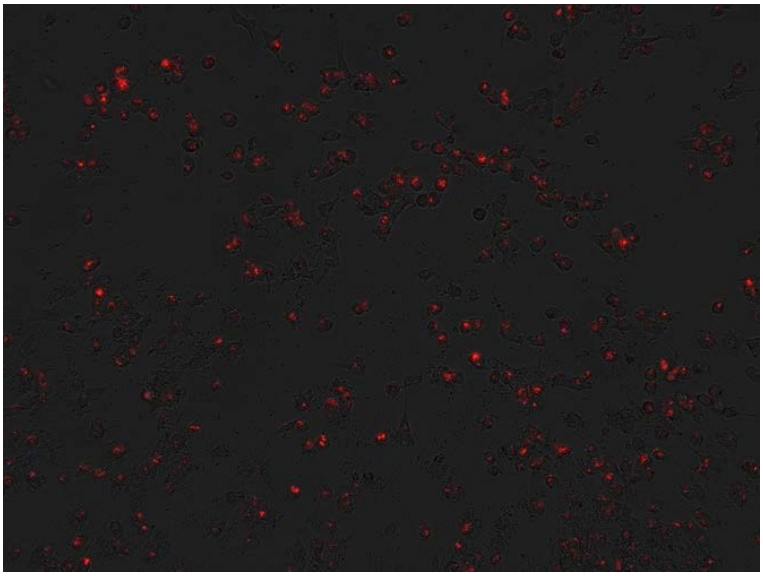
The matcha doses of 50 µg/ml and 100 µg/ml were non-toxic and did not have any significant harmful effects on zebrafish embryos, as determined by the results of all the toxicity assessments. Consequently, these concentrations were used in the xenograft experiments in an attempt to uncover the anticancer properties of matcha.

Red Fluorescence Labeling of MDA-MB-468 And MDA-MB-231 Breast Cancer Cells

In preparation for starting the xenograft model, triple negative human breast cancer cells (MDA-MB-468 and MDA-MB-231) were stained successfully. The incubation time was optimized until adequate and uniform fluorescence labeling was obtained for both cell lines. Visualization and imaging were done using an EVOS M5000 fluorescence microscope. The RFP fluorescent filter with excitation/emission spectra of 531/593 was used to examine the fluorescence of the cells as the CM-Dil fluorescent dye has an excitation/emission of 553/570 nm maxima. Figure 8 shows a representative image of fluorescently labeled MDA-MB-231 cells. Figure 8-A shows the cancer cells emitting red fluorescence after staining. In contrast, in Figure 8-B the image was merged with a bright field image to show the cells emitting the red fluorescence.



(A)



(B)

Figure 8. MDA-MB-231 breast cancer cells labeled with red fluorescent CM-Dil dye. Cancer cells were visualized under the EVOS M5000 microscope with red fluorescent protein filter (A) and merged with the bright field image (B).

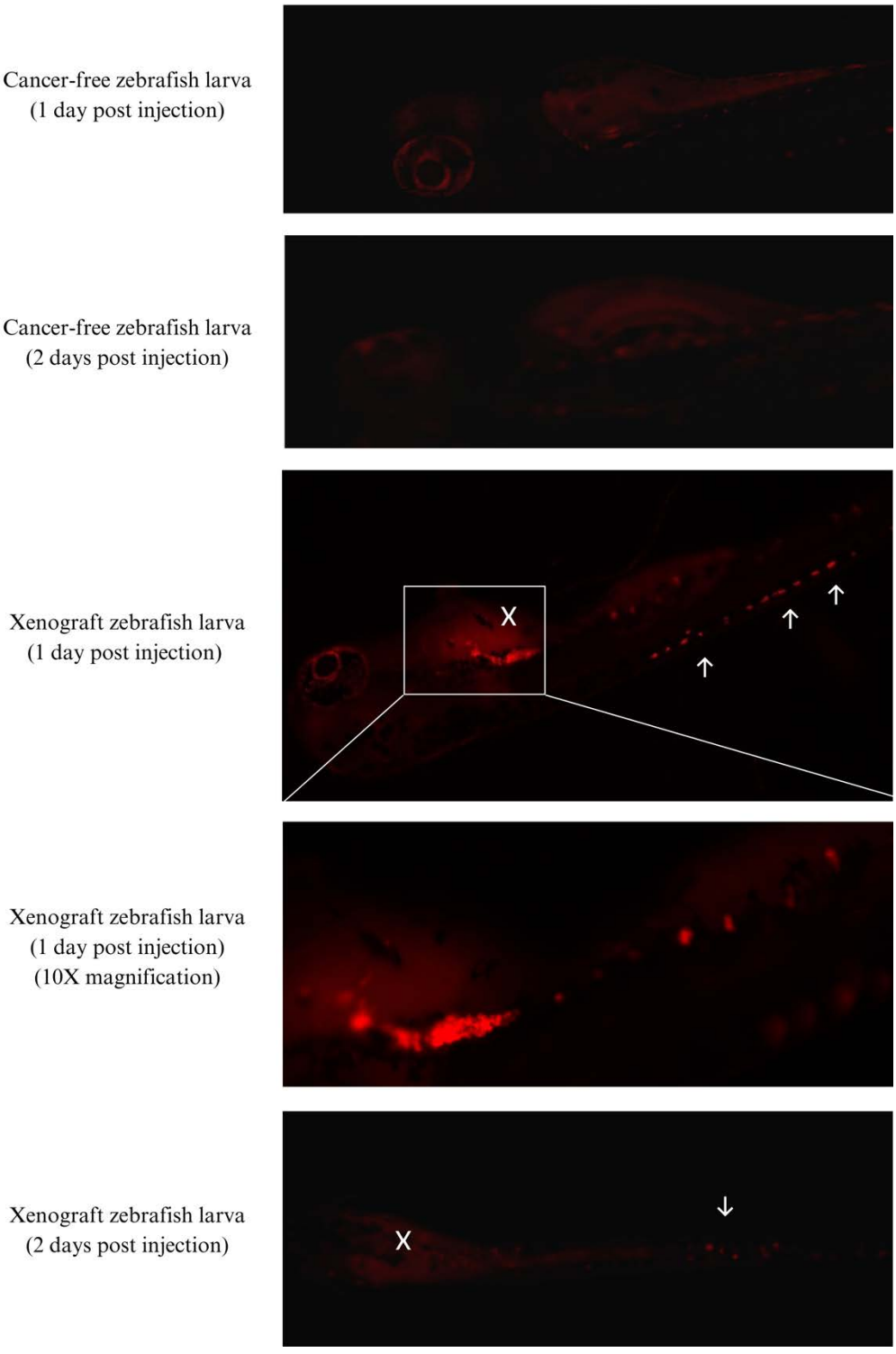
Establishing a Zebrafish Xenograft Model

Triple negative breast cancer cells (MDA-MB-468 and MDA-MB-231) were successfully xenografted into 48 hpf zebrafish larvae. Zebrafish larvae

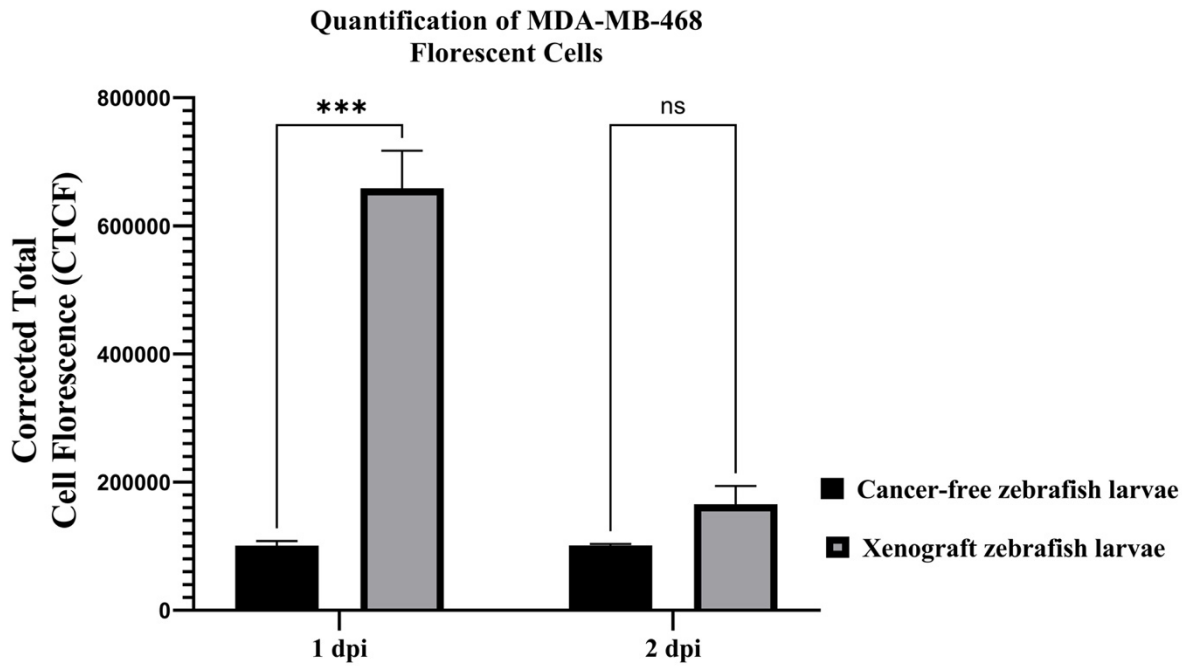
xenotransplanted with MDA-MB-468 (Figure 9-A) and MDA-MB-231 (Figure 10-A) were imaged from one to two days post injection (dpi) and compared to cancer-free control larvae. The figures show the differentiation between the embryos' autofluorescence and the cancer cells' attachment. At 1 dpi, both cell lines labeled with CM-Dil red fluorescent dye adhered near the injection site, which is the yolk sac. The cancer cells' injection site (white X) showed the most tumor mass in both cell lines. Both cell lines were able to metastasize to other regions of the zebrafish larvae indicated by the white arrows. The cancer cells formed clusters of cells that have the ability to metastasize to different sites, mainly to the tail area. We also noticed a significant decline in MDA-MB-468 cancer cells florescence at 2 dpi. Moreover, MDA-MB-231 cells were able to significantly proliferate and grow in the zebrafish larvae, as seen by increased cancer cells at 2 dpi. The survival rate of the embryos after injection was constantly above 70%, which is an acceptable survival rate for embryos used in an invasive procedure such as microinjection.

Quantification results revealed a successful xenotransplantation of both TNBC cells into 48 hpf zebrafish larvae. MDA-MB-468 showed high fluorescence at 1 day post injection, revealing successful attachment and homing of cancer cells inside the zebrafish (Figure 9-B). However, the fluorescence decreased at two days post injection, leveling near the cancer-free control. On the other hand, MDA-MB-231 cancer cells showed high fluorescence at 1 day post injection, indicating attachment of the cancer cells inside zebrafish larvae (Figure 10-B). At two days post injection, significantly higher fluorescence was observed in xenograft larvae ($p < 0.05$) compared to cancer-free control, reflecting proliferation and growth of MDA-MB-231 cancer cells inside the xenograft zebrafish larvae.

Establishing zebrafish xenograft model with MDA-MB-468 TNBC cells



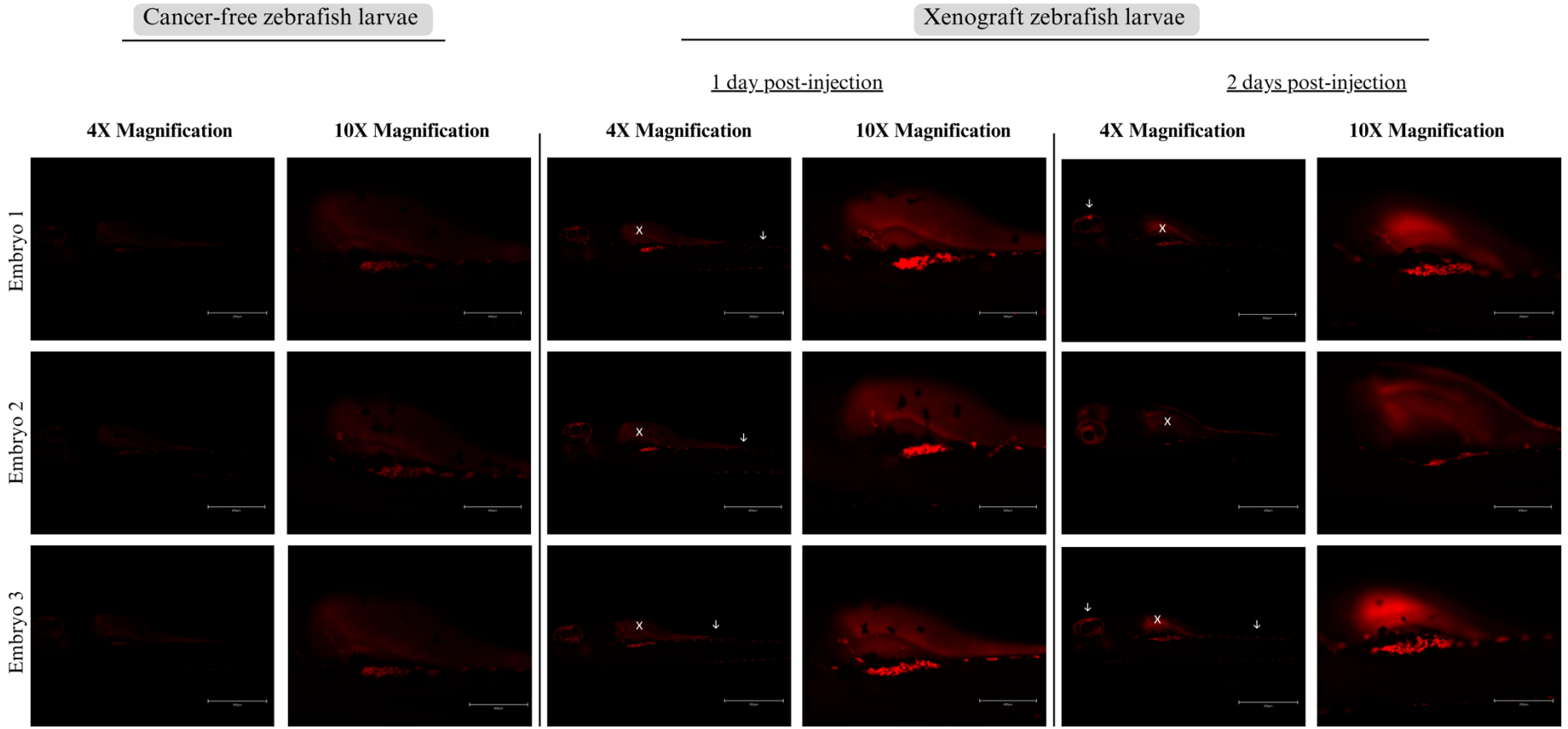
(A)



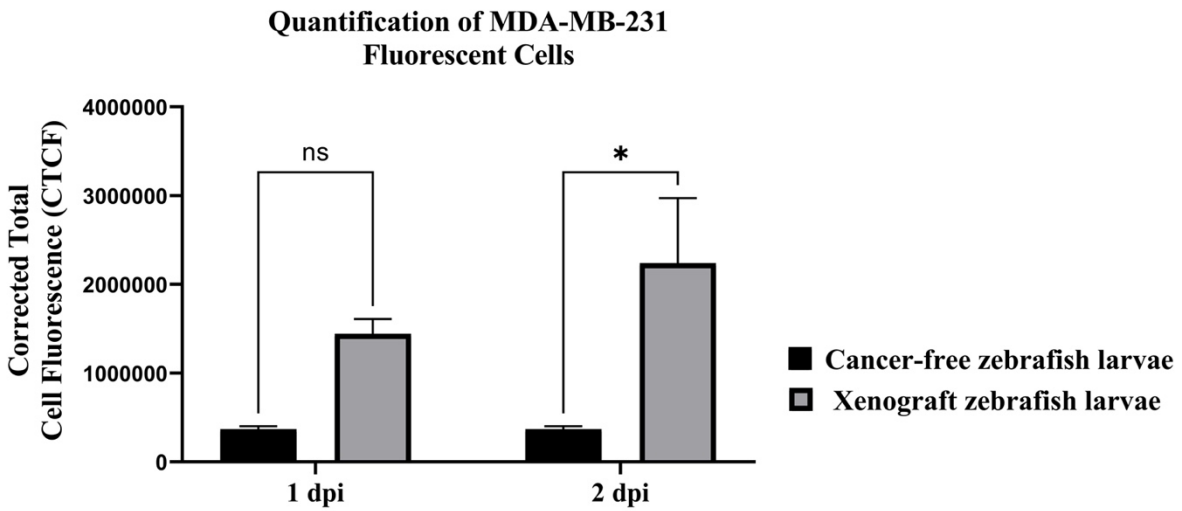
(B)

Figure 9. Zebrafish xenograft model injected with MDA-MB-468 cells at 2-days post fertilization (48 hpf). (A) Images of zebrafish embryos xenotransplanted with MDA-MB-468 cell line were taken over a period of 1- and 2-days post injection; the tumor cell mass was compared to an un-injected negative control embryo. All Images are at 4X magnification unless otherwise stated. (B) Quantification of MDA-MB-468 florescent cancer cells inside the xenograft zebrafish compared to the cancer-free control using ImageJ software. Abbreviations: dpi, days post injection.

Establishing zebrafish xenograft model with MDA-MB-231 TNBC cells



(A)



(B)

Figure 10. Zebrafish xenograft model injected with MDA-MB-231 cells at 2-days post fertilization (48 hpf). Images of 3 zebrafish embryos xenotransplanted with MDA-MB-231 cell line, taken over a period of 1 and 2 dpi; the tumor cell mass was compared to 3 un-injected negative control embryos. Abbreviations: TNBC; triple negative breast cancer. (B) Quantification of MDA-MB-231 florescent cancer cells inside the xenograft zebrafish compared to the cancer-free control using ImageJ software. Abbreviations: dpi, days post injection. *$p<0.05$.

Xenograft Zebrafish Model Exposed to Matcha

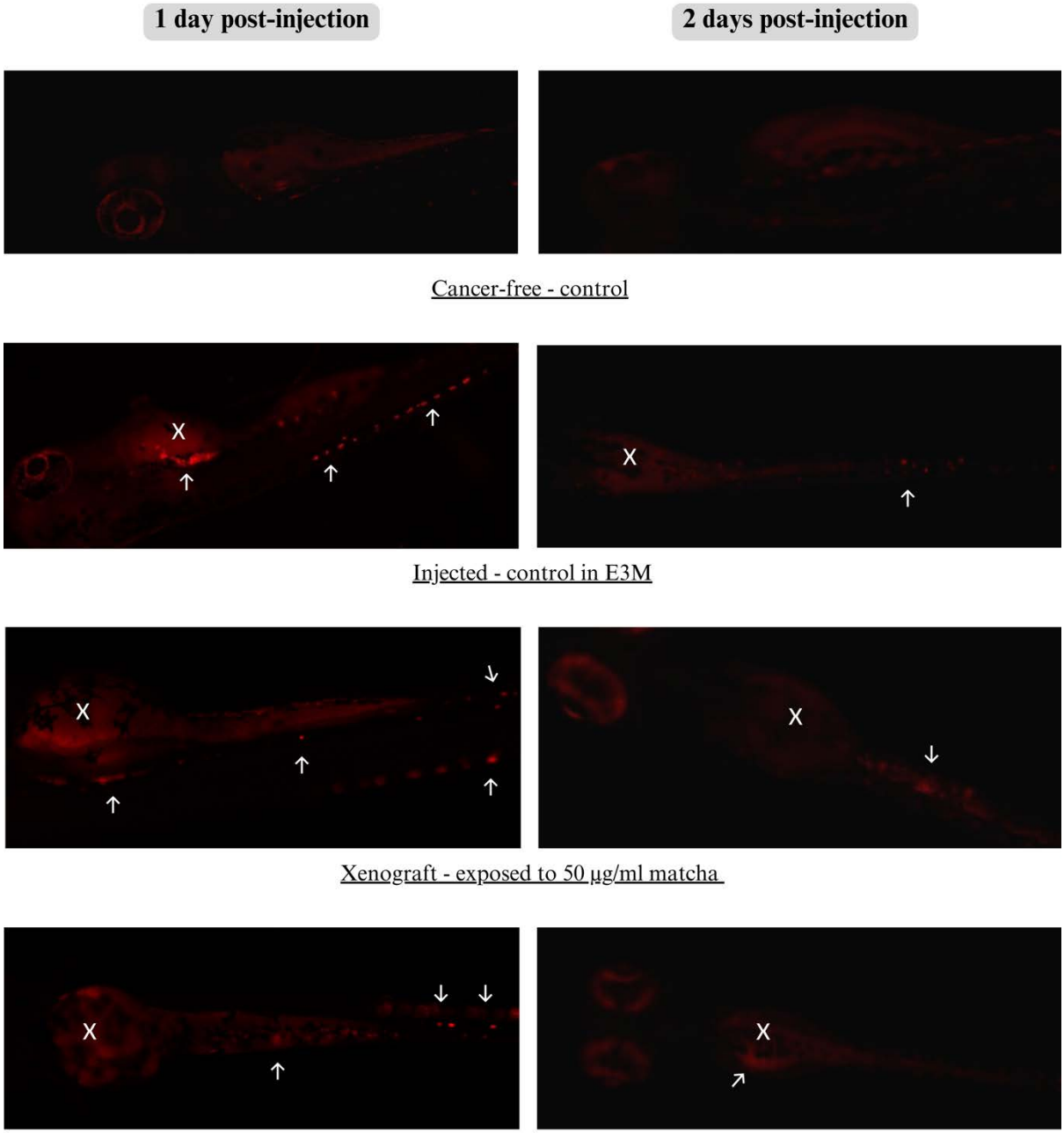
Xenograft zebrafish larvae were exposed to matcha concentrations at 50 and 100 µg/ml simultaneously with injected xenograft control larvae to assess the anticancer effect of matcha. Figure 11-A and Figure 12-A show the xenograft larvae exposed to matcha at 1- and 2-days post injection, compared to injected control maintained in E3M and cancer-free control. The injection site, which is the yolk sac, is indicated by a white X mark, while white arrows mark the fluorescently labeled cancer cells. As seen in Figure 11-A, matcha exposure at 50 µg/ml reduced the mass size of the MDA-MB-468 tumor cells at 1 dpi; however, higher suppression was seen at 100 µg/ml matcha. On the other hand, at 1 dpi, migration and metastasis of the cancer cells to the tail area were still visible with exposure to 50 µg/ml but were less visible at 100 µg/ml of matcha. Compared to the injected control, a dose-dependent decrease in tumor mass size and was seen with higher matcha concentrations at 1 dpi. An overall decrease in cancer cells was seen at 2 dpi, indicating failure of the cancer cells to survive in the zebrafish larvae.

Figure 12-A shows the effect of matcha exposure on the tumor size and metastasis of MDA-MB-231 breast cancer cells. After exposing the xenograft larvae to matcha concentrations at 50 and 100 µg/ml, the tumor size reduced slightly at 1- and 2-days post injection. The reduction in the tumor size was noted to be dose-dependent, as higher reduction was seen at 100 µg/ml compared to 50 µg/ml matcha concentration. MDA-MB-231 cells were also able to form clusters of cells and metastasize to the tail region. Matcha exposure at 50 µg/ml did not reduce metastasis of the tumor cells compared to the control; however, 100 µg/ml of matcha was able to reduce the metastasis of the cells to the tail region. The suppression of metastasis was seen more

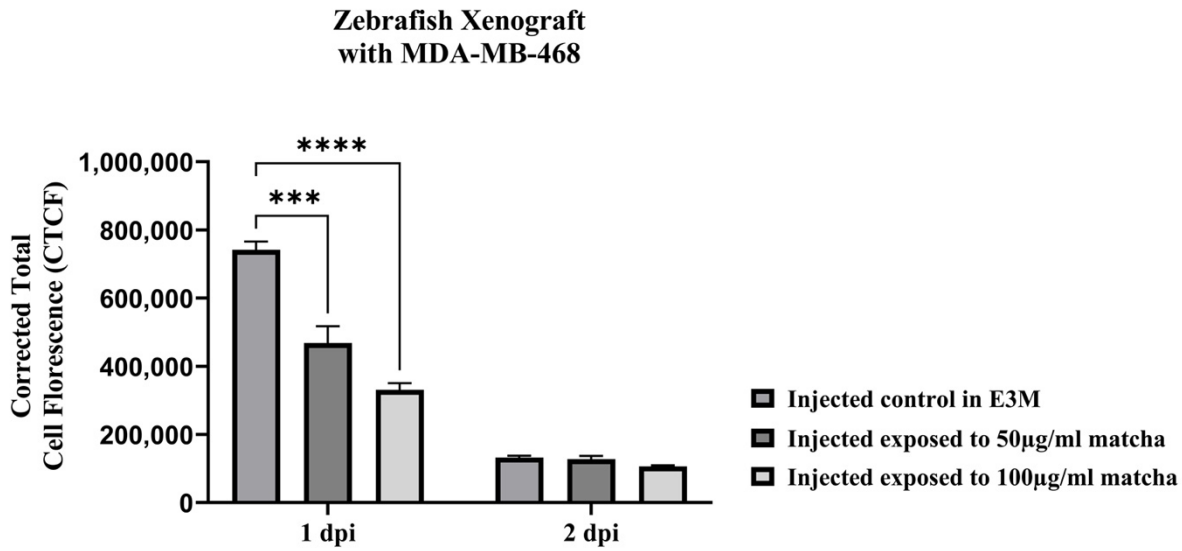
clearly after 2 days of injection, related to longer matcha exposure duration. At 2 dpi, we observed a general higher background fluorescence in the yolk sac area, which reflects the increased proliferation of tumor cells and growth inside the yolk sac area, also confirmed by quantification data.

Quantification results of fluorescently labeled cancer cells in the xenograft zebrafish control not exposed to matcha showed significantly higher fluorescence compared to cancer-free control. This xenotransplanted cell fluorescence was significantly high in both MDA-MB-468 and MDA-MB-231 cells, but the intensity was more profound in the former ($p < 0.01$ and $p < 0.5$, respectively). At 1 dpi, MDA-MB-468 xenograft larvae exposed to matcha concentrations at 50 and 100 µg/ml showed a trend towards reduction in the tumor mass size in a dose-dependent manner (Figure 11-B). At 2 dpi, all xenograft zebrafish larvae showed a profound reduction in fluorescence, including larvae exposed to matcha. On the other hand, MDA-MB-231 matcha-exposed xenograft larvae showed lower fluorescence at 1 dpi compared to xenograft control in a dose-dependent manner (Figure 12-B). At 2 dpi, high tumor growth and proliferation was seen in all xenograft larvae, however, matcha-exposed larvae showed a less substantial increase.

Effect of matcha on MDA-MB-468 TNBC cells zebrafish xenograft

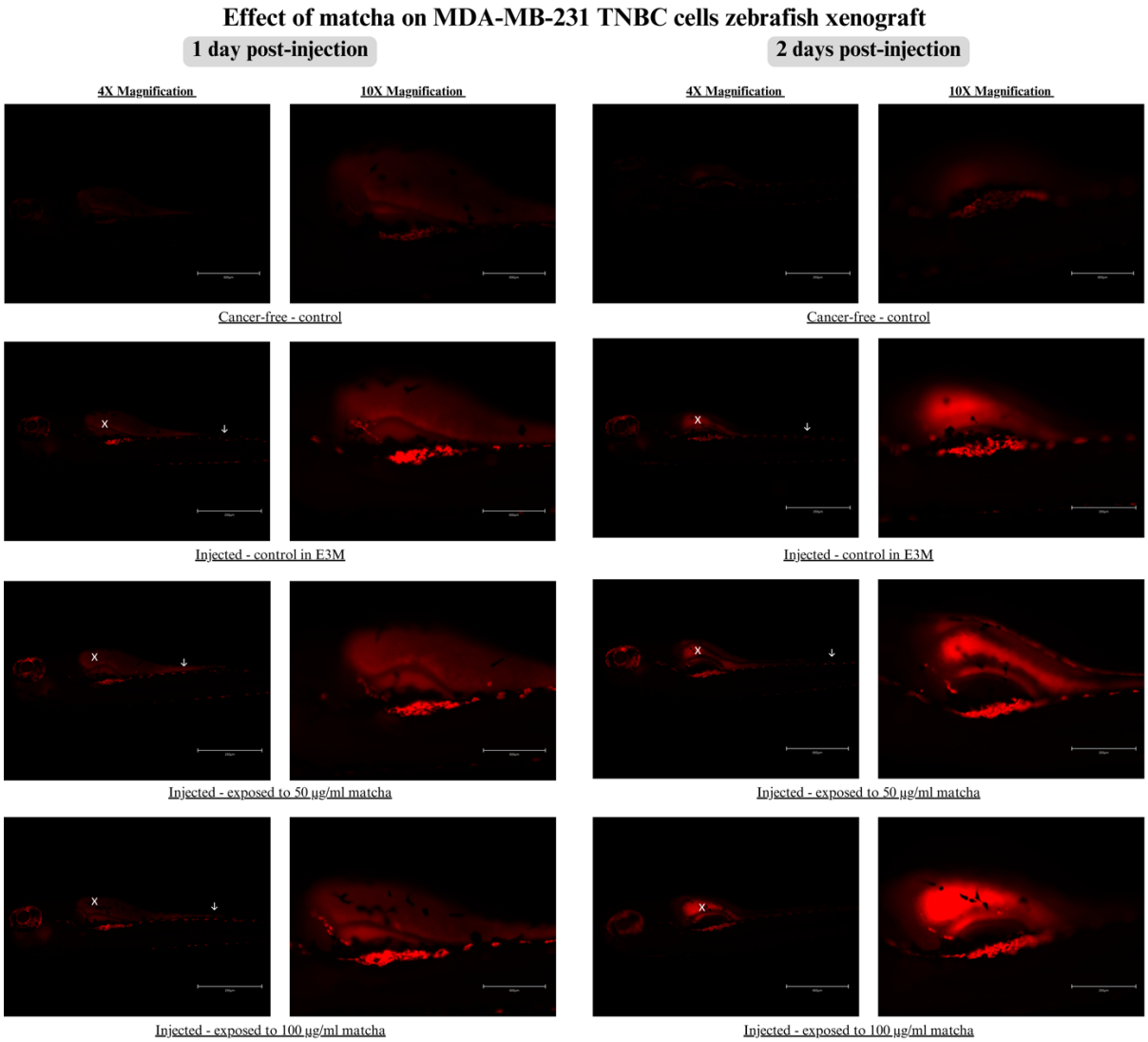


(A)

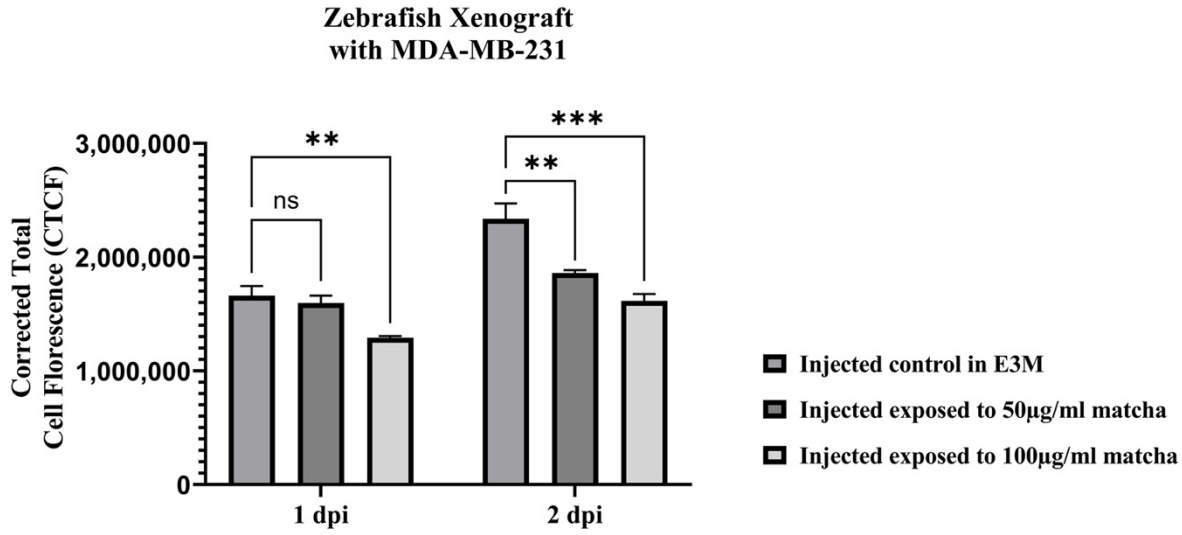


(B)

Figure 11. Zebrafish xenograft model injected with MDA-MB-468 cells at 2-days post fertilization (48 hpf) and exposed to 50 and 100 µg/ml matcha. (A) Images of zebrafish embryos xenotransplanted with MDA-MB-468 cell line exposed to 50 and 100 µg/ml of matcha were taken over a period of 1- and 2-days post injection, and the tumor cell mass and metastasis were compared to an injected control embryo maintained in E3M. (B) Quantification of MDA-MB-468 florescent cancer cells inside the xenograft zebrafish compared to the cancer-free control using ImageJ software. Abbreviations: dpi, days post injection; E3M, Embryo medium. *$p<0.05$, **$p<0.01$.


Effect of matcha on MDA-MB-231 TNBC cells zebrafish xenograft
1 day post-injection
2 days post-injection
4X Magnification
10X Magnification
4X Magnification
10X Magnification
Cancer-free - control
Cancer-free - control
Injected - control in E3M
Injected - control in E3M
Injected - exposed to 50 µg/ml matcha
Injected - exposed to 50 µg/ml matcha
Injected - exposed to 100 µg/ml matcha
Injected - exposed to 100 µg/ml matcha

(A)


Zebrafish Xenograft
with MDA-MB-231
Corrected Total
Cell Florescence (CTCF)
3,000,000
2,000,000
1,000,000
0
ns
**
**
***
1 dpi
2 dpi
Injected control in E3M
Injected exposed to 50µg/ml matcha
Injected exposed to 100µg/ml matcha

(B)

Figure 12. Zebrafish xenograft model injected with MDA-MB-231 cells at 2-days post fertilization (48 hpf) and exposed to 50 and 100 µg/ml matcha. Images of zebrafish embryos xenotransplanted with MDA-MB-231 cell line exposed to 50 and 100 µg/ml of matcha were taken over a period of 1 and 2 dpi; and the tumor cell mass and metastasis were compared to an injected control embryo maintained in E3M. (B) Quantification of MDA-MB-231 florescent cancer cells inside the xenograft zebrafish compared to the cancer-free control using ImageJ software. Abbreviations: dpi, days post injection; E3M, Embryo medium. *$p<0.05$.

Chapter 5: Discussion

Matcha has been gaining popularity recently and is consumed frequently in drinks and confectionary sweets (Baba, Takihara, Sagesaka, & Kaneko, 2019; Unno et al., 2019). The numerous beneficial health effects of matcha further increased its popularity. However, the effect of matcha on cancer is an interesting field of study that still requires more investigation. Our study is the first to explore the anticancer effect of matcha using zebrafish xenograft model. We first identified the optimal concentration of matcha that caused no significant toxic effect on the zebrafish by assessing multiple toxicity assessments. These include the toxic effect on the cardiovascular and neuromuscular systems and the zebrafish larvae's survival and hatching rates.

The survival of the larvae exposed to matcha was affected by doses higher than 200 µg/ml. Since our study is the first experimental study to assess the effect of matcha on the normal development of zebrafish, our findings are novel. Previous studies examined the effect of different types of teas on the development of zebrafish (Q. Zhang et al., 2022). However, due to the uniquely higher content of bioactive ingredients in matcha compared to other types of tea (Ashihara & Suzuki, 2004; Horie et al., 2017), our results were different. Matcha being in a powdered form allows more release of these bioactive ingredients into the dissolved solvent. Matcha was found to release 3 times more bioactive molecules and polyphenols once dissolved in water compared to other popular teas, including leaf-form green tea (Fujioka et al., 2016). Previous research showed that green tea might have toxic effects on the normal development of zebrafish, but the concentrations they used started from 500µg/ml of green tea (Q. Zhang et al., 2022), which is ten times higher than the lowest concentration used in our study (50 µg/ml), leading to observed toxic effects.

The hatching of the zebrafish larvae typically takes place after 48 to 52 hours of fertilization; however, matcha doses higher than 150 µg/ml significantly decreased the percentage of larvae hatching from their chorions even after the 72 hours post fertilization time point. Hatching reflects the normal development of zebrafish embryos, and disruption in the hatching rate is considered a toxic side effect of the treatment. Although bioactive molecules in matcha, such as theanine, caffeine, and catechins have been associated with multiple health-promoting, disease-preventing, and anticancer effects in the zebrafish model (Kochman et al., 2020; J. Xu et al., 2022; B. Zhao et al., 2022; Y. Zhao et al., 2022; Zong, Chen, Li, & Zhang, 2021), high concentrations of these molecules can disrupt normal development of zebrafish. This is also due to the high sensitivity of zebrafish to any subtle changes in their environment (e.g., PH, conductivity) (Bauer, Mally, & Liedtke, 2021).

Further investigation of the cardiovascular health of the zebrafish was performed by assessing five parameters assessed at the dorsal aorta: the average blood flow velocity, vessel diameter, shear stress exerted on the vessel, the flow rate, and the heartbeat. Similar to hatching rate results, matcha concentration higher than 150 µg/ml was associated with a significant increase in the blood flow velocity compared to the control. Meanwhile, 200 µg/ml of matcha caused a significant increase in shear stress. Abnormally high blood flow velocity within a major blood vessel, such as the dorsal aorta, suggests vascular abnormalities or defects of the heart muscle (Brown, Samsa, Qian, & Liu, 2016). Deviations from the normal values for shear stress (4.04 ± 0.73 dynes/cm^2), representing the frictional force of flowing blood on the blood vessel and heart walls, indicate cardiac toxicity (Eisa-Beygi et al., 2018; Salman & Yalcin, 2020). The two values of blood flow velocity and shear stress are clinically and statistically related, since the calculations for shear stress include the average blood flow velocity

value (Benslimane et al., 2020). Moreover, all the other parameters were not significantly different from the control, and all fell within the normal ranges for dorsal aorta cardiac parameters of a 3 days old (72 hpf) zebrafish larva, according to a validation study by Benslimane et al. (Benslimane et al., 2020), heartrate: 120-180 bpm, vessel diameter: 15.84 ± 2.5 µm, and flow rate: 5.1 ± 1.1 nL/ml. Matcha concentrations below 150 µg/ml were not associated with cardiotoxic side effects on zebrafish larvae.

To confirm the effect of matcha on the neuromuscular system of zebrafish, locomotion assessment was conducted by tracking the movement of individual larvae at 96 hours post fertilization while exposing them to alternating light/dark cycles. Zebrafish embryos gain a well-developed neuromuscular system at 96 hours, which is the time when locomotion assessment is conducted (Drapeau et al., 2002). Our results showed that the movement of the larvae was significantly higher after exposure to 100 and 150 µg/ml of matcha. The higher matcha concentrations showed a dose-dependent increase in the average distance moved by the larvae, which could be due to the higher caffeine concentration the larvae were exposed to, leading to more agitation and alertness in the zebrafish during the light cycles (Clayman & Connaughton, 2022). Caffeine enhances cognitive function, increases alertness and focus, and improves memory (Fredholm, Bättig, Holmén, Nehlig, & Zvartau, 1999; Smit & Rogers, 2000). Also, compared to other types of tea, matcha is composed of higher caffeine content due to picking only young leaves in the process of creating matcha. Moreover, theanine, a major bioactive compound found in matcha, promotes memory, reduces anxiety, reduces oxidative stress, and protects nervous system function in zebrafish (B. Zhao et al., 2022). However, the doses used in mentioned study (33.3 µg/ml) are much higher than the doses used in our experiments (2.3% of matcha's dry weight, i.e., 2.3 µg and 1.15 µg in the 100 and 50 µg/ml concentrations, respectively), which could explain not

seeing the therapeutic effect of theanine in our matcha-exposed groups. These results were also reflected in the calculated total distance moved by all embryos of the experimental group, where 150 µg/ml matcha concentration showed a statistically significant increase, and the dose-dependent effect was still seen as an increase in the total distance at higher matcha concentrations.

After selecting the safe matcha concentration to test its beneficial anticancer effects, we established different zebrafish xenograft models using MDA-MB-468 and MDA-MB-231 triple negative breast cancer cells. Both cell lines are a subtype of breast cancer cell line that was derived from the pleural effusion of a patient with metastatic breast cancer. MDA-MB-468 cells present multiple characteristics of breast cancer, such as uncontrolled cell division, invasion, and metastasis (Welsh, 2013). MDA-MB-231 cell line is also known to be highly aggressive, invasive, and poorly differentiated (Chavez, Garimella, & Lipkowitz, 2010; Liu, Zang, Fenner, Possinger, & Elstner, 2003). It is also known to be more metastatic in *in vivo* models (Xiao et al., 2021) and usually represent a late-stage cancer model (Amaro et al., 2016). The xenograft zebrafish models were successfully established, indicated by adhering to the xenotransplanted breast cancer cells near the yolk sac and spreading to other areas after 1 day of injection for both cell lines.

MDA-MB-468 breast cancer cells preferred to adhere near the site of injection, which is the yolk sac and formed clusters once they attached to the home site. The cancer cells were also seen to migrate in clusters to different sites in the zebrafish embryo, such as the tail, which was also seen in previous studies (Berens, Sharif, Wellstein, & Glasgow, 2016; Rebelo de Almeida et al., 2020). After two days of injection, we observed a significant reduction in cell fluorescence, indicating that MDA-MB-468 cancer cells does not remain florescence after 30 hours which is the

peak florescence time of the CM-Dil dye used. Previous studies also tried to establish a xenograft zebrafish model with MDA-MB-468 breast cancer cells. One study was not successful due to the development of severe cardiac edema in > 50% of the xenograft larvae (Berens et al., 2016), while other studies were successful (Berens et al., 2016; Rebelo de Almeida et al., 2020; Schwarz-Cruz y Celis et al., 2020; Shahi Thakuri et al., 2020). Studies demonstrated the high adherence and metastasis of MDA-MB-468 cells inside the zebrafish larvae, similar to our results. One study showed that, compared to multiple other types of TNBC cells, MDA-MB-468 was identified as having the highest metastasis potential in the zebrafish larvae (Rebelo de Almeida et al., 2020). Upon exposure to matcha, tumor cells were seen to decrease in size, in a dose-dependent manner, where 100 µg/ml matcha concentration reduced fluorescence signals more than 50 µg/ml. Two days after injection, we observed a reduction in the tumor size at the yolk sac for all xenograft larvae, but a few cancer cells were still fluorescent in the tail area. This was confirmed by fluorescence quantification. More extended observation periods are needed to confirm the long-term effect of matcha exposure on the metastatic activity and tumor growth of MDA-MB-468 breast cancer cells.

MDA-MB-231 cancer cells were also followed up on day 1 and 2 after injection, and the xenograft model was shown to be successful. Tumor cells adhered to the injection site, similar to MDA-MB-468, and could metastasize to other regions in the zebrafish larvae, specifically to the tail area. Previous studies also demonstrated that MDA-MB-231 cells were able to metastasize to the tail area within 24 hours post injection (J. Xiao et al., 2022). Other studies also successfully established a zebrafish xenograft model using this cell line (Fan et al., 2021; Guo, Fan, & Pei, 2020; Xiao et al., 2021; J. Xiao et al., 2022), and they similarly showed high intravasation as well as extravasation (Berens et al., 2016; Xiao et al., 2021). Exposure to matcha reduced tumor

mass size of MDA-MB-231 cancer cells in a dose-dependent manner without statistical significance. However, the metastasis of the tumor cells was more visibly reduced after matcha exposure, where captured images showed that matcha concentration at 100 µg/ml was more able to suppress metastasis of the cells after 1 day of injection compared to 50 µg/ml, and this suppression was more substantial after 2 days of injection. This indicates that MDA-MB-231 cells require higher concentrations and more extended periods of exposure to matcha to show a significant anticancer effect. Further investigation is needed to examine the potential higher tumor growth and metastasis suppression at more extended matcha exposure periods to MDA-MB-231. Molecular markers of breast cancer prognostic and/or therapeutic value should be considered, such as hormone receptors, p53 proteins, and HER-2 oncogene (Banin Hirata et al., 2014).

When administered at safe levels that cause no adverse side effects in zebrafish embryos and larvae, matcha and its components exhibit multiple health-promoting functions in the zebrafish animal model. One study examined the effect of Catechins, which are especially abundant in matcha, on zebrafish's oxidative damage and apoptosis in their early development stages. The results confirmed a protective antioxidant effect of Catechins by increasing the expression and activity of the antioxidant enzymes in zebrafish (Y. Zhao et al., 2022). No previous studies examined the effect of a whole matcha extract on the cancer xenograft zebrafish; however, components of matcha were shown to reduce cancer in the zebrafish model (Shahi Thakuri et al., 2020; J. Xu et al., 2022). The tea pigment Theabrownin is a bioactive compound found in all green teas, and it has been reported to have an effect against human cancers *in vitro* (Jin et al., 2018; Xu et al., 2020). In the zebrafish animal model, a study showed that Theabrownin could inhibit tumor growth, increase senescence and

increase apoptosis of hepatocellular carcinoma cells xenotransplanted into zebrafish larvae (J. Xu et al., 2022). Additionally, Quercetin is another compound abundant in matcha, where the aqueous extract of matcha contains 1.2 mg/mL, only marginally higher than in traditional green tea (1.1 mg/mL) (Kochman et al., 2020). Quercetin was proven to be highly effective in suppressing the migration of triple negative breast cancer cells in zebrafish (Shahi Thakuri et al., 2020), and its combination with EGCG, the most abundant catechin in matcha, resulted in higher suppression of tumor growth in cell cultured triple negative breast cancer cells (Schroder et al., 2019). This indicates that the holistic effect of matcha may exert more powerful anticancer effects due to the interaction and effects of multiple bioactive compounds. The effect was tested for the first time in our study by observing the effect of matcha on tumor mass size and metastasis in zebrafish xenograft model. Our results showed a reduction in the tumor size of the MDA-MB-468 cells and suppressed metastasis of the MDA-MB-231 cells xenotransplanted into zebrafish.

Our study has multiple strengths, including using whole matcha and not individually isolated components, which is more relevant to the human consumption of matcha and can help guide recommendations for human cancer prevention. Also, we administered matcha for 96 hours post fertilization, which is a sufficiently long period of time enough to mimic a human daily consumption lifestyle. Our study is also strong for the novelty of being the first research to test the anticancer effect of matcha using an animal model. Additionally, we are the first study to determine the safe, non-toxic dose of matcha suitable for zebrafish to explore its beneficial anticancer effects and successfully determined the NOAEL to be 50 and 100 µg/ml.

Limitations

This study was limited by a few factors, including the complexity of maintenance zebrafish, due to being very sensitive to any subtle changes in temperature, PH, conductivity, and light-dark cycles (Bauer et al., 2021). Any change in these aspects affects the health of the fertilized embryos and may lead to the absence of any embryo production or the production of low-quality zebrafish embryos after fertilization. Moreover, the fluorescence signal detected from the CM-Dil dye starts to diminish after 30 hours of staining (Andrade, Seabrook, Johnston, & Hay, 1996), which may have affected the observed results at 2 dpi. Finally, in our study we were only able to follow up the xenograft_zebrafish for only 2 days post injection due to the ethical approval obtained.

Conclusion

Previous literature showed that green tea might be toxic to zebrafish development (Q. Zhang et al., 2022). Therefore, an emphasis on the toxicity assessment is essential to eliminate potential deviations from the actual results regarding the anticancer effect of matcha tea on the xenograft zebrafish larvae. We were able to test the toxicity of matcha and found that concentrations between 50 and 100 µg/ml were safe and did not cause significant adverse effects in zebrafish. We also successfully established a zebrafish xenograft model using two human triple negative breast cancer cell lines, MDA-MB-231 and MDA-MB-468. The anticancer effect of matcha using zebrafish as an *in vivo* model was successfully tested, and a trend towards reduction in tumor mass size and metastatic abilities was seen after exposure to matcha in a dose-dependent manner. Both cell lines used in this study are highly invasive, metastatic, and resistant to anticancer drugs, and the observed effect of significantly lower proliferation upon exposure to matcha for only two days is noteworthy and triggers further investigation. Extended observational period after xenotransplantation of cancer

cells is needed to confirm the long-term anticancer effect of matcha and to derive recommendations for human matcha consumption for cancer prevention.

Chapter 6: Appendix

QU-IBC Approval

Qatar University
Institutional Bio-safety Committee

To: Maha AlAsmakh, PhD **May 11, 2022**
Qatar University

Ref: Project Titled Developing Zebrafish as an in vivo model to screen medicinal plants for anti-cancer activity in human breast cancer
Grant: QUCP-CHS-2022-483

Dear Dr. Maha AlAsmakh, PhD,
We would like to inform you that your application along with supporting documents provided for the above proposal have been reviewed by QU-IBC, and having met all the requirements, has been granted approval.

Please note that QU-IBC approval is contingent upon your adherence to the following QU-IBC Guidelines:

- Ensuring compliance with QU Safety Plans and applicable national and international regulations.
- Ensuring experiments that require prior IBC approval are not conducted until IBC approval is obtained and making initial determination of containment levels required for experiments.
- Notifying the IBC of any changes to other hazardous material experiments previously approved by the IBC.
- Reporting any significant problems, violations of QU Safety Plans and applicable regulations/ guidelines, or any significant research-related accidents and illnesses to the QU-IBC. Also, ensuring personnel receive appropriate orientation and specific training for the safe performance of the work.

Your research approval No. is: **QU-IBC-2022/014**. Please refer to this approval number in all your future correspondence pertaining to this research.

Best wishes,
Chairperson, QU-IBC

Institutional Bio-safety Committee
(IBC)
Office Of Academic Research

Approved Date: April 24, 2022
Qatar University Institutional Biohazard Committee (QU-IBC)

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