Universidade de Aveiro Departamento de Química
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ANA FILIPA GOMES AIRES

EFEITOS DOS COMPOSTOS CITOSTÁTICOS EM EMBRIÕES DE PEIXE ZEBRA

EFFECTS OF CYTOSTATIC COMPOUNDS ON ZEBRAFISH EMBRYOS

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Bioquímica Clínica, realizada sob a orientação científica do Doutor Marcelino Miguel Oliveira, Investigador Auxiliar do Departamento de Biologia da Universidade de Aveiro e da Doutora Maria de Lourdes Pereira, Professora Associada com Agregação do Departamento de Ciências Médicas da Universidade de Aveiro.

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Palavras-chave

Compostos citostáticos, peixe zebra, biomarcadores bioquímicos, biomarcadores comportamentais, metotrexato, 5-fluorouracilo

Resumo

Os compostos citostáticos são um grupo de substâncias quimioterapêuticas com diferentes mecanismos de ação cuja utilização tem tido um aumento significativo. Estes compostos, usados principalmente no tratamento do cancro são, após a sua administração, excretados via urina e/ou fezes dos pacientes em tratamento e posteriormente libertados para o ambiente através de efluentes hospitalares e/ou esgotos domésticos. Devido à ineficiência das estações de tratamento de águas residuais, estes compostos chegam ao ambiente afetando animais não alvo como, por exemplo, os peixes. O peixe zebra é um modelo biológico muito utilizado no estudo do desenvolvimento de vertebrados, modelação de doenças humanas como o cancro e avaliação do efeito de contaminantes ambientais. A sua utilização em muitos campos de investigação tem vindo a aumentar, dadas as vantagens que apresenta comparativamente a outros modelos tradicionais como por exemplo os ratinhos (e.g. facilidade de manipulação, manutenção laboratorial, similaridade genética com humanos). Esta dissertação está dividida em três capítulos onde inclui uma introdução geral, um artigo de revisão e o trabalho experimental, e teve como principal objetivo avaliar as respostas fisiológicas, bioquímicas e comportamentais provocadas por dois compostos citostáticos, metotrexato e 5-fluorouracilo, individualmente e em combinação, no peixe zebra, após exposições de curta duração (120h) a uma gama de concentrações, incluindo concentrações ambientalmente relevantes. Foram detetados efeitos de ambos os compostos citostáticos nos parâmetros avaliados. Com o metotrexato estas diferenças foram detetadas predominantemente na concentração mais elevada (1000 $\mu g.L^{-1}$), enquanto com o 5-fluorouracil e com a mistura há diferenças em todas as concentrações testadas. Ambos os fármacos mostraram provocar stress nos organismos e de um modo geral provocaram alterações ao nível cardíaco e na cauda do peixe zebra. Este trabalho irá contribuir para a compreensão das consequências da libertação destes compostos para o ambiente, identificar respostas biológicas mais sensíveis e potenciais mecanismos de ação tóxica.

Keywords

Cytostatic drugs, zebrafish, biochemical biomarkers, behavioral biomarkers, methotrexate, 5-fluorouracil

Abstract

Cytostatic drugs are a group of chemotherapeutic substances with different mechanisms of action and with a significant increase of usage. These drugs, mainly used in cancer treatment are, after their administration, excreted via the urine and/or feces of the patients being treated, and further released into the environment through hospital wastewater and/or domestic sewage. Due to the inefficiency of wastewater treatment plants, these compounds reach the environment affecting non-target animals, such as fish. Zebrafish is a biological model widely used in the study the development of vertebrates, the modeling of human diseases, such as cancer, and the evaluation of the effect of environmental contaminants. Its use in many fields of research has been increasing, given the advantages it presents in comparison to other traditional models such as mice (e.g. ease of handling, laboratory maintenance and genetic similarity with humans). This dissertation is divided into three chapters which includes a general introduction, a review article and the experimental work, and the principal aim of this work was to evaluate the physiological and biochemical as well as behavioral responses provoked by two cytostatic compounds, methotrexate and 5-fluorouracil, individually and in combination, in zebrafish, after short exposures (120h) to a range of concentrations, including environmentally relevant concentrations. Effects of both cytostatic compounds were detected in the evaluated parameters. With methotrexate these differences were detected predominantly at the highest concentration (1000 $\mu g.L^{-1}$), while with 5-fluorouracil and the mixture there were differences in all tested concentrations. Both drugs have been shown to cause stress in the organisms and in general have caused changes in the heart level and tail of the zebrafish. This work will shed some light for the understanding of the consequences of the release of these compounds into the environment, to identify more sensitive biological responses and potential mechanisms of toxicity.

Abbreviations

5-FU	5-Fluorouracil
AChE	Acetylcholinesterase
CAP	Capecitabine
CAT	Catalase
CDDP	Cisplatin
CP	Cyclophosphamide
DHFR	Dihydrofolate reductase
dTMP	Deoxythymidine monophosphate
dUMP	Deoxyuridine monophosphate
FdUMP	Fluorodeoxyuridine monophosphate
FET	Fish Embryo Toxicity
FUDP	Fluorouridine diphosphate
FUDR	Fluorouridine
FUMP	Fluorouridine monophosphate
FUTP	Fluorouridine triphosphate
GST	Glutathione S-transferase
IF	Ifosfamide
LDH	Lactate dehydrogenase
MTX	Methotrexate
ROS	Reactive Oxygen Species
THF	Tetrahydrofolate
TS	Thymidylate synthase
TYMK	Thymidylate kinase
TYMP	Thymidine phosphorylase
TYMS	Thymidylate synthase
UK	Uridine kinase
UMPS	Uridine monophosphate synthetase
WWTP	Wastewater treatment plant

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Figure 17. Evaluation of CAT activity after zebrafish exposure to 5-fluorouracil. (*p<0.05)

Figure 18. Heart beats measured at 48 hpf in zebrafish larvae after exposure to the combination of methotrexate and 5-fluorouracil. Values are expressed in beats per minute. (*p<0.05)

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Figure 20. Representative specimens of zebrafish pictures after exposed to the combination of methotrexate and 5-fluorouracil, with 100x ampliation, taken during the FET test. **A-** Control; **B-** Edema and Malformation ([MIX]= 10 $\mu g.L^{-1}$); **C-** Edema ([MIX]= 10 $\mu g.L^{-1}$);

D- Edema ([MIX]= 10 μg.L^{-1}); **E-** Tail deformation ([MIX]= 10 μg.L^{-1}); **F-** Tail malformation ([MIX]= 10 μg.L^{-1}); **G**– Edema ([MIX]= 100 μg.L^{-1}); **H-** Edema and Tail deformation ([MIX]= 1000 μg.L^{-1}).

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Thesis structure and framing

This thesis, conducted under the scope of an interdisciplinary cooperation between CESAM, CICECO, Department of Chemistry, Department of Biology and Medical Sciences from Aveiro University, is based on two papers:

- Ana Aires, Diana Gomes Moreira, Amadeu M.V.M. Soares, Maria L. Pereira, Miguel Oliveira. Effects of cytostatic substances in environment (to be submited).
- Ana Aires, Maria L. Pereira, Miguel Oliveira. Effects of Methotrexate, 5-Fluorouracil and this mixture on zebrafish embryos (to be submited).

It also includes a general introduction focused on the problem of cancer, the different types of cytostatic agents, their presence in the environment, the assessment of their effects, and information about zebrafish. A general discussion is also presented where all the data is integrated and discussed, and future perspectives resulting from this work are presented.

Chapter I: General Introduction

1. Introduction

Cancer is one of the main causes of death in the whole world and its incidence has been increasing every year as a result of the current lifestyle and increased longevity of the human population (1-3). Cancer is described as a malignant tumor or abnormal cell growth. This abnormal growth results from the continuous unregulated proliferation of cells and the acquisition of metastatic properties, despite the restriction of nutrients and space, causing effects that are generally detected in tissues with fast-dividing cells (e.g. stomach, blood cells, cells lining the mouths) (4,5). Prostate, breast, lung and colon/rectal cancer are the cancers with the highest prevalence rates and in 2020, 1247588 new cases were registered in women and 1444949 in men in Europe, represented in **Figure 1**, distributed in percentages (6,7). Upon cancer diagnosis, there is the need to resort to medical treatments which may include radiotherapy, chemotherapy and/or surgery. Another type of treatment is epigenetic therapy, which may help target disseminated cancer progenitor cells. Chemotherapy is the most widely used treatment for cancer, and consists on the use of cytostatic drugs to treat

the cancer and, as a result of increasing incidence of cancer diseases, there is an increase in the consumption of cytostatic drugs (pharmaceuticals used in the chemotherapy) (3,8,9). These biologically active substances (and/or their metabolites), after administration to patients and after the usual metabolic processing occurs, are released via hospital effluents or domestic wastewater after excretion in the urine and/or feces, and thus having the potential to affect the non-target organisms (10).

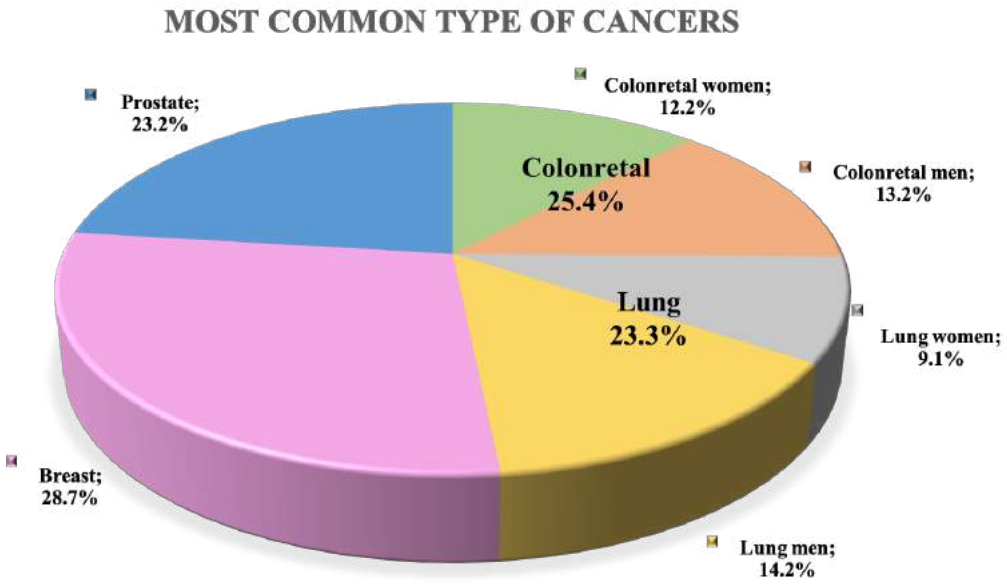

Figure 1. The most common cancers diagnosed in women and men in 2020, distributed in percentages (Adapted from (6)).

2. Cytostatic drugs

Cytostatic drugs, also known as anticancer or antineoplastic drugs, are a group of synthetic and natural chemotherapeutic substances with different mechanisms of action (8,11). They were used for the first time in 1940, when antifolate drugs and nitrogen mustards were applied to cancer treatment, and salicylic acid was detected in the environment for the first time in 1970 (4,12). Overall, the main purpose of cytostatic drugs is to cause of decease of rapidly growing cells such as those found in cancer tumors in order

to reduce the disease. These substances act differently, depending on their type (4). However, in general they interfere with DNA and RNA, preventing DNA from making copies of itself, leading to an inability of the cell to reproduce (11). These drugs can cause delayed toxic effects on aquatic life and human health in long-term exposure, representing a potentially serious environmental risk (13).

Apart from the hospital environment, potential sources of cytostatic drugs are the pharmaceutical industry, pharmacies, veterinary medicine, home care facilities and waste treatment plants (**Figure 1**) (13). Thus, these drugs can also attack non-target cells, dividing cells, causing severe cytotoxic effects such as mutagenic, teratogenic, genotoxic and carcinogenic side-effects (12). To enhance the clinical effectiveness of these drugs in chemotherapy and to increase the scientific knowledge on the toxic side effects of chemotherapeutic drugs it is necessary to understand the molecular basis of drug interaction (9).

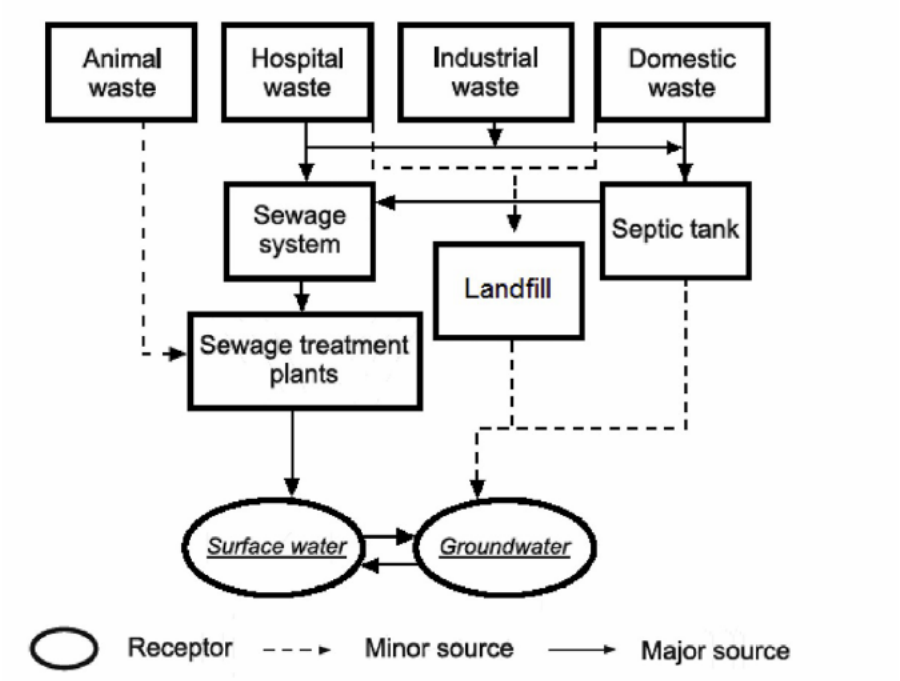

Figure 2. Different sources and pathways of cytostatic pollution in water (from (11)).

Cytostatic drugs can be classified into the following groups (**Table 1**) according to the mechanisms: i) platinum complexes, ii) alkylating agents, iii) intercalating agents, iv) antimetabolites, v) antitumor antibiotics, vi) topoisomerase inhibitors, vii) mitotic spindle inhibitors, viii) monoclonal antibodies and iv) protein kinase inhibitors (9,12). Although the

principal mechanism is the same for cytostatic drugs, different substances show different properties and modes of action, such as the inhibition of processes that occur at higher rates in cancer cells, the inhibition of substances necessary for cellular replication or interruption of different processes (4).

Table 1. Different groups of cytostatic drugs, examples of pharmaceuticals and their mechanism of action.

Group of cytostatic	Mechanism of action	Pharmaceutical	References
Platinum complexes	Action consists in causing intrastrand and inter-strand cross-links in DNA, particularly including two adjacent guanine adenine or two adjacent guanine bases.	Cisplatin, Carboplatin	(14)
Alkylating agents	Direct interaction with cellular DNA avoiding its replication	Chlorambucil, Cyclophosphamide	(4)
Intercalating agents		Amonafide, Pyrazoloacridine	
Antimetabolites	Interference with DNA precursors and cellular metabolism resembling cellular metabolites such as pyrimidine and purine nucleotide bases and folic acid	Methotrexate, 5-Fluorouracil, Gemcitabine	(4)
Antitumor antibiotics	Inhibition of cell wall synthesis or nucleic acid synthesis, inhibition of ribosome function or cell membrane function, inhibition of folate metabolism and thus change the DNA of cancer cells to keep them from growing and multiplying	Bleomycin, Doxorubicin	(15)
Topoisomerase inhibitors	Interference with the action of topoisomerases enzymes I and II, which help to separate the DNA strands for them be copied, thus inhibiting the replication of DNA and interfering with transcription and replication by causing DNA damage	Etoposide, Epirubicin	(16)
Mitotic spindle inhibitors	Inhibition of polymerization dynamics of the microtubules blocking the transition from metaphase to anaphase	Vincristine, Docetaxel	(5)

Table 1 (Continuation). Different groups of cytostatic drugs, examples of pharmaceuticals and their mechanism of action.

Group of cytostatic	Mechanism of action	Pharmaceutical	References
Monoclonal antibodies	Blockage of ligand binding and subsequent signal interruption. They also be used to affect the immune response of the host to the cancer cells and to deliver chemotherapy molecules to malignant cells via drug conjugates	Rituximab, Alemtuzumab	(17)
Protein kinase inhibitors	Inhibition of the activation of specific proteins, including receptors responsible for oncogenese, due to their specificity and selectivity	Imatinib, Ponatinib	(18)

Methotrexate and 5-Fluorouracil are two commonly used cytostatic drugs in chemotherapy (9). In this perspective, understanding the molecular basis of the interaction of these drugs is fundamental in order to enhance the clinical effectiveness of treatment (9).

2.1. Cytostatic drugs in environment

Pharmaceuticals are present in residential, agricultural and industrial waste streams and receiving coastal waters (13). Many pharmaceuticals used in human and veterinary medicine, such as chemotherapeutic drugs, are not completely metabolized and are most often directly discharged into the sewage system, without any specific control after being administered by out-patients or in hospitals (10). The drugs go to the sewage system after being administered, and released by patients through urine or feces, partially in their original chemical form or as metabolites. The current treatment processes implemented in wastewater treatment facilities are not completely effective in degrading/removing these hazardous compounds and thus, these substances may reach surface waters and threaten the aquatic biota (2).

The ingestion of toxic substances, such as cytostatic drugs, can result in a stressing condition for organisms, often non-target animals, in a trophic transfer through the food web, and risks to human health through seafood intake (19).

Nowadays pharmaceuticals are recognized as important emerging environmental contaminants with cytostatic drugs being detected in the aquatic environment at different concentrations, depending on the type of drug (12,13). Cytostatic drugs and their metabolites have low biodegradability and can reach the water cycle through wastewater treatment plant (WWTP) effluents (20). Although their concentration in effluents and influents of WWTPs are normally within the ngL^{-1} range, the risks associated to the chronic exposure to these drugs are a great concern due to their effects on organisms, biodiversity preservation and environmental safety (20).

The use of cytostatic drugs has increased due to an escalating incidence and early diagnosis of cancer leading to the detection of many parent compounds, metabolites and their residues in the environment (21). Cytostatic drugs, which induce significant organ toxicity and are potentially carcinogenic to humans are also found in hospital effluents, river water and estuarine water (13). Reported levels in different countries, are between 13.7 and 70.2 ngL^{-1} in effluents and 0 to 10.3 ngL^{-1} in influent, for antineoplastic agents (8). However, these values depend on the type of drug and vary in each country. Methotrexate and 5-Fluorouracil are two of the mainly used cytostatic drugs in the treatment of cancer and, consequently, more present in the environment. Methotrexate has been detected in Germany with values of 0.5 ngL^{-1} in effluents and 0.6 ngL^{-1} in influents but in Spain these values are 66 ngL^{-1} in effluents and 308 ngL^{-1} in influents (22). Another example is 5-Fluorouracil which has values of 23 ngL^{-1} and 0.21 ngL^{-1} in effluents in the United Kingdom and Spain, respectively (2,8).

3. Assessment of effects

Toxicity tests are important to assess the effect of drugs on living cells and to understand their mechanisms of action. In these tests it can be possible to check how safe a substance is but also to characterize the possible toxic effects it can cause. In toxicity tests, biological models are chosen according to the substance in study, the route of exposure, and the sensitivity of the organism. The assessment of the biological responses of organisms to pollutants is an extremely useful tool in toxicology, allowing the evaluation of the sensitivity of the organism upon drug exposure (23). Toxicity tests can assess acute toxicity, sub-acute toxicity and chronic toxicity tests. Acute toxicity tests are characterized by short term

assessment and evaluation of potential hazard test substance or consequences of a test substance (23). Sub-acute toxicity tests are used to determine organs affected by different dose levels. Lastly, chronic toxicity tests determine the affected organs and check whether the drug is potentially carcinogenic or not even on short exposures (23).

The duration of exposures is important to establish the mode of action or the mechanism for a toxic effect, to ensure safety of new chemicals for use, such as pesticides, drugs, or food additives and to establish a dose response curve (23).

3.1. Biochemical Biomarkers

Biomarkers are molecular, physiological, biochemical and behavioral responses indicating modifications in a biological system due to a potential hazard (24). They can offer a more complete and relevant information about the potential impact of a substance on the health of animals (19). Furthermore, biomarkers are clinically useful when they can supplement the clinical diagnosis, help in evaluation of treatments and monitoring of the disease, and predicting prognosis and health outcome (25).

Exposure to contaminants may lead to an unbalance between reactive oxygen species (ROS) production and elimination, inducing direct effects on physiological performance and health status of organisms (19). Oxidative stress is a common mechanism of toxicity and can lead to massive protein oxidation and degradation and lipid peroxidation, causing cell injury or cell death. It occurs because some substances can catalyze the formation of ROS. Some cellular protection mechanisms, such as the activity of antioxidant enzymes (catalase (CAT)), allow detoxification of ROS before they cause oxidative damage. Glutathione S-transferase (GST) is considered an important biomarker of defense related to detoxification mechanisms, namely cellular detoxification against xenobiotics and noxious compounds as well as against oxidative stress (19,26).

Lactate dehydrogenase (LDH) is used to detect cell damage, and is the first isoenzyme ever used as biomarker to detect specific organ damage (25).

In addition to oxidative stress, neurotransmission is often affected by toxic substances, and there is also an often association between behavioral alterations and neurotransmitter effects (27). Acetylcholinesterase (AChE) activity is used to measure the effects on neurotransmission after exposure to drugs or other foreign substances. AChE has

some advantages as biomarker (e.g. easy to measure, sensitive, and exhibits a link to health adverse effects) (27).

3.2. Fish Embryo Toxicity Test and Behavior alterations

The Fish Embryo Toxicity Test (FET) with the zebrafish (*Danio rerio*) is an alternative to the standard acute fish toxicity test and has been standardized, optimized and validated in OECD TG 236 as a test to assess toxicity on embryonic forms of fish (28).

Acute toxicity tests with animals, designed to determine the dose that will produce mortality or serious toxicological effects, when given, are the most common tests for environmental hazard and risk assessment (28). They can help in environmental hazard identification and risk assessment of products like chemicals, food additives, plant protection products, biocides, and effluents. However, the FET is an alternative to the fish acute test, allowing the detection of teratogenicity, neurotoxicity, genotoxicity and mutagenicity, as well as various forms of endocrine disruption (28,29).

FET consists on the exposure of newly fertilized zebrafish embryos to a test chemical for 96h. Every 24h alterations are observed, such as 1) coagulation of fertilized eggs, 2) lack of somite formation after 24 hours of exposure, 3) lack of detachment of the tail bud from the yolk sac after 48 hours and 4) lack of heartbeat after 48 hours. At the end of the exposure period, the acute toxicity is determined based on significant differences in any of the four observations recorded (28).

In addition to FET, to determine the mortality and the physiological alterations in the organism when exposed to certain drug concentrations, behavioral effects can also be analyzed, often using automated observation and tracking software. In fish, parameters like swimming behavior can be evaluated, reflecting the potential changes at a lower biological level (28).

4. Zebrafish

Zebrafish (*Danio rerio*) is a tropical freshwater fish belonging to the family Cyprinidae (30). This species has been used for studying vertebrate development and to model human diseases (31). It has also been predominantly used as a model in developing

biologic and molecular genetics, but their value in toxicology as well in drug discovery has been recognized (32). The use of zebrafish as an experimental model has increased steadily in many fields of research (30). Zebrafish is an ideal animal model for toxicological research since it can be used at all stages from early development embryos to juveniles and adults allowing morphological, biochemical and physiological studies, where the objective is to identify adverse effects of chemicals (30). There are many attributes of zebrafish that are favorable to the study of cancer pathogenesis and the search for drug targets and chemotherapeutics such as husbandry factors, small-molecule screening that can be performed on a physiologically intact vertebrate disease model. In addition, zebrafish is well established as a chemical carcinogenesis model (33,34). Thus, this animal is an attractive model for the developmental origins of health and disease and transgenerational studies due to some advantages such as: i) high fecundity, one pair of adult fish is capable of laying 200-300 eggs in one morning; ii) short generation time, mature adults develop in about three/four months; iii) external fertilization and development; and easy maintenance and breeding (**Figure 2**) (31,34). They have numerous other advantages such as: iv) small size, adults and embryos have only approximately 5 cm and 5 mm at 7 days post-fertilization (d.p.f.), respectively (35); v) embryos are relatively large and initially transparent, which allow visualization of any developmental abnormalities associated with exposure and direct observation of organ function can be easily performed (31,34,35); vi) husbandry is cheaper than for rodents (34); vii) its genome has been fully sequenced, 71.4% of human genes are related to zebrafish genes (34). Zebrafish proteins are reasonably similar to their human counterparts, particularity within functional domains as well in the tissues and organs (30,35). Human disease models for screening efficacy have been developed in zebrafish in a wide range of therapeutic areas (cancer, inflammation and metabolic diseases, infections and cardiovascular diseases) (35); viii) zebrafish is also used for safety pharmacology in the identification of the feasibly possible potential safety of drugs selected for human evaluation (35).

Exposure of zebrafish, during sensitive stages of its development, to environmental chemicals, such as cytostatic drugs dumped in the sewage, can have consequences for adult life (31).

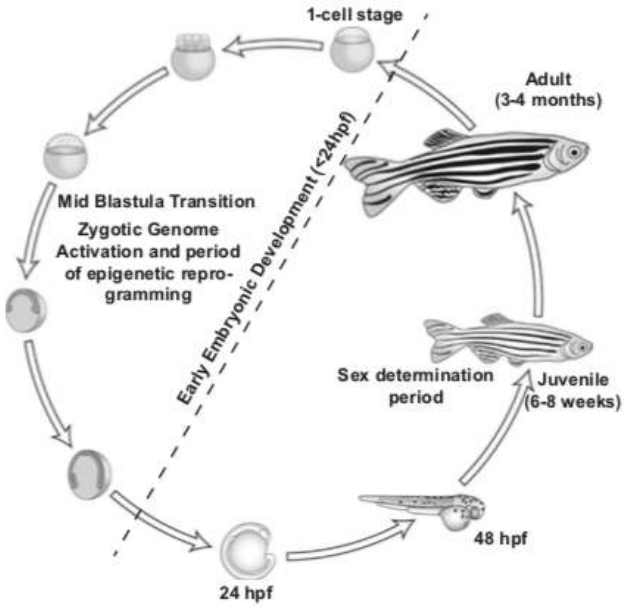

Figure 3. Main steps in the development of zebrafish (from (31)).

5. Aims and conceptual framework of the thesis

Considering the increased release of cytostatic drugs in the environment, the aim of this study was to assess the potential effects of these substances on non-target organisms, trying to understand/elucidate potential modes of action. The interaction of cytostatic substances and the hazard for the environment was also assessed.

Thus, this dissertation is structured in three main chapters. After a first chapter with a general introduction, the second chapter consists of an article review on the effects of cytostatic substances in the environment. The third and last chapter lists the effects of Methotrexate, 5-Fluorouracil and the mixture of both on zebrafish embryos, and the results are discussed accordingly.

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Chapter II

Article Review: Effects of cytostatic substances and their environmental levels

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Abstract

Cytostatics are a group of chemotherapeutic substances with a variety of mechanisms of action and have had a significant increase in their use. These drugs used mainly in the treatment of cancer, are released into the environment after their administration through hospital wastewater or domestic sewage, as a result of excretion through the urine and/or feces of patients being treated. Once in the environment, and due to the inefficiency of wastewater treatment plants, they can affect non-target animals, such as fish. This problem is alarming, since drugs can combine among themselves, causing more serious effects and becoming an even greater risk to the environment. The present study aimed to provide a critical review on the environmental levels reported in the aquatic environment and their effects on fish.

Keywords

Cancer, cytostatic drugs, zebrafish, environmental levels

1. Introduction

Nowadays, there is a notable increase in the use of drugs, including cytostatics. The use of this type of pharmaceutical is mainly due to the fact that the incidence of cancer has been increasing as a result of the current lifestyle and increased longevity of the human population (1). Considering the biological nature of these drugs and growing number of people suffering from cancer, cytostatic substances have become a major environmental concern (2).

Cancer is a group of diseases that results from the continuous unregulated proliferation of cells and the acquisition of metastatic properties (3,4). These cells invade normal tissues and organs and, ultimately, can spread throughout the body (3). During this process, cancer cells modify their metabolism to meet the requirements of cellular proliferation, facilitating the uptake and conversion of nutrients (5). Prostate, breast, lung and colon/rectal cancers, are the types with the highest prevalence rates (3,6). To combat these diseases, cytostatic drugs have been used and administrated during chemotherapy (7).

These biologically active substances (and/or their metabolites), after administration to patients and metabolism, are excreted in the urine and/or feces and released into hospitals or domestic wastewater (8). These substances can be found on the surface, ground and drinking waters, as they are not efficiently removed during wastewater treatment plants and may affect non-target animals (9).

2. Pharmaceuticals in the environment

Pharmaceuticals are recognized as important emerging environmental contaminants (10). They are present in residential, agricultural and industrial waste streams and receiving coastal waters (10). Most pharmaceuticals used in human medicine, such as chemotherapeutic drugs, are not completely metabolized and are often directly discharged into the sewage system, with no specific control after being released by patients through urine or feces, in their original chemical form and/or as metabolites in out-patients or hospitals (8). These compounds are not effectively removed during wastewater treatment processes and, therefore, may reach surface waters and may threaten the aquatic biota (2,11).

The exposure to toxic substances, such as cytostatic drugs, can result in a stressful condition for the biota, and a trophic transfer through the food web. Therefore, there is potential that organisms at higher trophic levels may be at greater risk, suggesting risks to human health through seafood intake (12).

Hospitals effluents are the main source of emission of cytostatics in the aquatic environment (9). However, other potential sources of antineoplastic drugs may be reported outside the hospital environment, such as pharmaceutical industry, pharmacies, veterinary sectors, laundry facilities, home care facilities, and waste treatment plants can contribute to the release of these substances into the environment (**Figure 1**) (10).

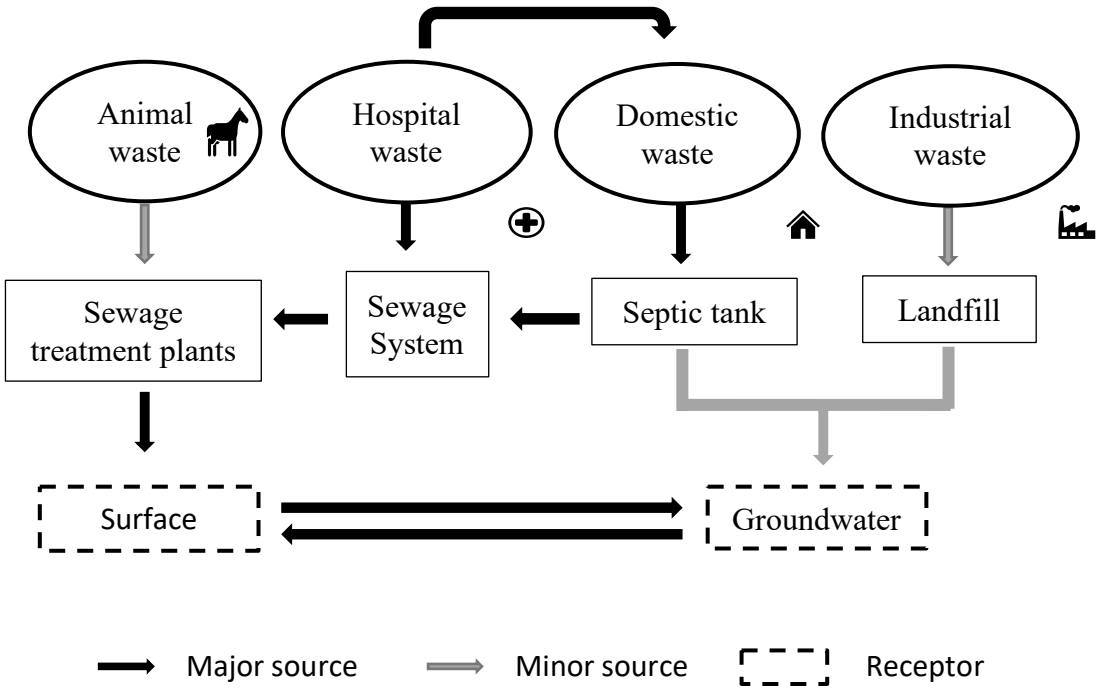

Figure 1. Different sources and pathways of cytostatic pollution in water (Adapted from (9)).

3. Cytostatic drugs

Cytostatics, also known as anticancer or antineoplastic agents, are a group of substances of synthetic and natural origin, with different mechanisms of action (9,13). These drugs, which can be classified into different therapeutic groups based on their mechanisms of action (e.g. alkylating agents, antimetabolites, antitumor antibiotics and topoisomerase inhibitors) are widely used in chemotherapy, the most common treatment for cancer (7). Thus, chemotherapeutic drugs are administered to interact with the tumor or cancer cells, aiming to prevent the growth of tumor cells and cell division, interfering in the signaling pathways of cellular genetic material (14). Cytostatic drugs and their metabolites are very reactive. They have a wide-range of action (cytotoxic, cytostatic and endocrine therapy) and can react with healthy non-target cells in cancer patients, which can cause uncontrolled cell damage (15). Currently, about 50 cytostatic substances are routinely used in chemotherapy in hospitals, the most commonly used are methotrexate (MTX), cyclophosphamide (CP), ifosfamide (IF), 5-fluorouracil (5-FU) and cisplatin (CDDP) (15,16). The modes of action of these substances vary, depending on the type of drug, but they generally kill fast-growing

cells such as those found in cancer. Antineoplastic agents (class I), endocrine therapy (class II) and immunostimulants and immunosuppressants (class III) (**Table 1**) are the three main classes of cytostatics currently used, classified under Anatomical Therapeutic Chemical (ATC) (13). They can also be classified into nine groups depending on the mechanism of action: i) antifolates, ii) antipyrimidines, iii) antipurines, iv) alkylant agents, v) platinum complexes, vi) antitumor antibiotics, vii) topoisomerase inhibitors, viii) antimitotic drugs and iv) microtubule-stabilizing agents (14,17).

Table 1. Classes of cytostatic drugs, respective groups and drugs. Mechanism of action of these classes.

Class of agent	Group	Drug	Mechanism of action	References
Class I (Antineoplastic agents)	Antifolates	Methotrexate	Inhibit essential biosynthetic processes or are incorporated into macromolecules (DNA or RNA); These drugs are either structural analogues for heterocyclic bases or agents interfering with folate metabolism; Inhibit main steps in the formation of purine and pyrimidine bases as well as nucleotides.	(18)
		Pemetrixed		
	Antipyrimidines	5-fluorouracil		(19)
		Capecitabine		
		Eniluracile		
		Hydroxyurea		
	Antipurines	6-mercaptopurine		(14)
		6-thioguanine		
Class II (Endocrine therapy)	Alkylant agents	Cyclophosphamide	Directly damages DNA by adding methyl or other alkyl groups onto nucleotide bases; Inhibits their correct utilization by base pairing leading to mutation, DNA fragmentation and inhibition of DNA replication and transcription.	(14)
		Ifosfamide		
		Melphalan		
		Chlorambucil		
	Platinum complexes	Cisplatin		
		Carboplatin		
	Antitumor antibiotics	Anthracyclines		
		Dactinomycin		
		Bleomycin		
		Adriamycin		
		Etoposide		
	Topoisomerase inhibitors	Camptothecine		
		Irinotecan		
		Topotecan		
Class III (Immunostimulants and immunosuppressants)	Antimitotic drugs	Vinblastine	Affect synthesis or breakdown of the mitotic spindle; Disrupt the cell division by either inhibiting the tubulin polymerization and the formation of the mitotic spindle.	(20)
		Vincristine		
	Microtubule-stabilizing agents	Paclitaxel		
		Docetaxel		

Cytostatic substances are frequently used in combination with other drugs such as opioids to achieve a higher therapeutic efficiency (9,16,17,18). However, these drugs are not cancer cell specific. They can affect cancer cells but also other cells, causing severe cytotoxic effects in humans and other animals, such as zebrafish. They can affect healthy tissues, and therefore, be dangerous (22). **Table 2** shows some studies with zebrafish exposed to different cytostatics with different concentrations, some of which are environmentally relevant, and the respective effects they caused. Cytostatic drugs normally act by completely inhibiting or blocking the replication of DNA in the tumor cell and, when possible, trigger cell death (9). To enhance the clinical effectiveness of these drugs in chemotherapy and increase the scientific knowledge on the side effects of chemotherapy, it is necessary to understand the molecular basis of drug interaction (7,9). These substances can be carcinogenic, embryotoxic, genotoxic, teratogenic and mutagenic and can cause disastrous damage to non-patients through drinking water or sea food which makes the need to understand the function and side effects of cytostatic drugs more important (9).

Table 2. Studies with zebrafish exposed to different cytostatics with different concentrations and the respective effects.

Cytostatic drugs	Concentrations	Essays or endpoints performed	Duration	Results	References
5-Fluorouracil	5, 10, 15 and 20 ngL^{-1}	- Mortality rate - Body lenght - p53 immunohistochemistry - Behavior	8 days	- Mortality in all tests (5-FU: 30-50%; LV: 20-30%; % higher in co-exposure); - Length increase in all tests; - Optical density decreased at the lowest tested concentration of 5-FU whereas with the highest concentration of 5-FU, 5 ngL^{-1} and 20 ngL^{-1} of LV was increased in the regions of the eyes and the intestine; - Total distance decrease with 5-FU and co-exposure and increase with LV; - Exposure to 5-FU, LV and co-exposure increased larvae velocity.	(23)
Leucovorin	5, 10, 15 and 20 ngL^{-1}				
5-Fluorouracil +Leucovorin	(5+15), (10+10), (15+5) ngL^{-1}				

Table 2 (Continuation). Studies with zebrafish exposed to different cytostatics with different concentrations and the respective effects.

Cytostatic drugs	Concentrations	Essays or endpoints performed	Duration	Results	References
Daunorubicin Pirarubicin Doxorubicin (DOX) Epirubicin DOX-liposome	1, 10, 20, 50, 100, 200 and 500 μM	- Toxicity and lethal dosage of zebrafish embryos - Heart rate, pericardial sac areas and heart tube looping	Three stages: 6-72 hpf 24-72 hpf 48-72 hpf	- Incomplete looping of the cardiac tube, pericardium edema, bradycardia in a dose-dependent manner leading to death; - Greatest heart defects in DOX exposure; - DOX-liposome reduced the effects on the heart; - Daunorubicin produced the least toxicity.	(24)
Imatinib mesylate (IM)	0.4 $mg.mL^{-1}$	- Ovarian histology and morphology - Sperm analysis - Breeding, embryo collection and fecundity and fertility determination - Gene expression analysis	30 days of exposure followed by a 30 days depuration period	- Irreversible suppression of folliculogenesis, concentration-dependent; - Reversible decrease in sperm density and motility; - Decreased fecundity and fertility; - Premature hatching; - Morphometric malformations; - Decreased expression of *vegfaa* and *igf2a* in testes and ovaries.	(25)
Imatinib mesylate-Brine Shrimp Nauplii (IM-BSN)	10 $\mu g.ml^{-1}$				
	Group 0: control Group 1: 1 IM-BSN + 2 BSN Group 2: 2 IM-BSN + 1 BSN Group 3: 3 IM-BSN				
Mitomycin C (MMC)	Viability assay: 0.01 and 0.05 $\mu g.ml^{-1}$ In vitro MN assay: 0.03 and 0.1 $\mu g.ml^{-1}$	- Viability assay - In vitro MN assay - In vitro and in vivo monitoring of nuclear buds, hypodiploidy and cytotoxicity	72h	- MMC, ETO and COL induced significant levels of cytotoxicity in HepG2 cells; - All induced the formation of MN in HepG2 cells; - Increase in the number of MN and hypodiploid nuclei with COL; - All induced the formation of MN in vivo; - Decreased mitotic index and induced hypodiploidy with ETO; - Increased diploidy, cytotoxicity at the highest concentration of COL.	(26)
Etoposide (ETO)	Viability assay: 0.2 and 1 $\mu g.ml^{-1}$ In vitro MN assay: 1 and 3 $\mu g.ml^{-1}$				
Cyclophosphamide (CP)	Viability assay: 2.5 and 15 $\mu g.ml^{-1}$ In vitro MN assay: 30 and 100 $\mu g.ml^{-1}$				
Demecolcine (COL)	Viability assay: 0.01 and 0.025 $\mu g.ml^{-1}$ In vitro MN assay: 0.03 and 0.1 $\mu g.ml^{-1}$				

Table 2 (Continuation). Studies with zebrafish exposed to different cytostatics with different concentrations and the respective effects.

Cytostatic drugs	Concentrations	Essays or endpoints performed	Duration	Results	References
Carboplatin (CarboPt)	0-4 hpf: 62.5-1000 μM 72 hpf: 2000-8000 μM	Fish Embryo Toxicity (FET)	96h	- PT more toxic and CarboPt less toxic in 0-4 hpf and 72 hpf; - Body deformities after 48 hours with 8 mM of CarboPt: body curvature and progressive disintegration of embryos to death (0-4 hpf); - Embryonic malformations and developmental abnormalities were assessed throughout the experiment, with no significant changes (72 hpf); - Cell viability assay: PT more toxic and CarboPT less toxic.	(27)
Irinotecan (IT)	0-4 hpf: 2-32 μM 72 hpf: 7-112 μM				
Doxorubicin (DOX)	0-4 hpf: 4-33 μM 72 hpf: 7-15 μM				
Paclitaxel (PT)	0-4 hpf: 0.5-8 μM 72 hpf: 0.002-0.188 μM				
Chloroquine (CQ)	0-4 hpf: 100-1000 μM 72 hpf: 50-250 μM				
Cisplatin (CP)	1, 10, 50, 100, 500 and 1000 μM	- Scanning ion-selective electrode technique (SIET): hair cells (Ca^{2+} influx) and ionocytes ($[H^+]$ gradients) - Survival rate - Hatching rate - Phenotype - Body length - Full body ions - Platinum content	96h	- Cisplatin impaired the function of hair cells (1 μM CP), the number of hair cells and the content of Cl^- body ions (10 μM CP); - Decreased ionocyte acid secretion; - Decrease in body ions of Na^+ and Ca^{2+} (50 μM CP); - Decrease in body length and ionocyte density (100 μM CP); - Decreased survival (500 μM CP); - Increased platinum accumulation in embryos with increased cisplatin concentration.	(10)

Table 2 (Continuation). Studies with zebrafish exposed to different cytostatics with different concentrations and the respective effects.

Cytostatic drugs	Concentrations	Essays or endpoints performed	Duration	Results	References
Cyclophosphamide (CP)	Cytotoxicity: 0.3-300 $mg.ml^{-1}$ Genotoxicity: 10-300 $mg.ml^{-1}$	- MTS assay - Comet assay - Cytokinesis block micronucleus (CBMN) assay	-	- CP and IF induced DNA strand break and genomic instability at concentrations higher or equal than 37.5 $mg.ml^{-1}$; - Both mixtures (MIX1 and MIX10) induced significant increase in the formation of DNA strand breaks; - Viability of zebrafish cells reduced after 72h exposure to CP or IF; - Viability of zebrafish cells was slightly lower after exposure to MIX10; - CP induced statistically significant increase in the micronuclei frequency at 150 and 300 $mg.ml^{-1}$; - Increase in the frequency of micronucleated cells was observed only at highest tested concentration of IF.	(28)
Ifosfamide (IF)					
Mixture: Cyclophosphamide (CP) + Ifosfamide (IF) + 5-Fluorouracil (5-FU) + Cisplatin (CDDP)	MIX1: [CP]=12 + [IF]=10 + [5-FU]= 0.09 + [CDDP]= 0.6 $(mg.ml^{-1})$ MIX10: [CP]=120 + [IF]=100 + [5-FU]= 0.9 + [CDDP]= 6 $(mg.ml^{-1})$				
Triptolide (TP)	0.41, 0.6, 0.8, 1, 1.23, 3,7 $\mu mol.L^{-1}$	- Acute toxicity assay - Cardiovascular toxicity assay	72h	- GA, TP and taxol showed highest acute lethality, with 50% lethal concentration around 1 $\mu mol.L^{-1}$; - Missing tails and severe pericardial edema were observed in MPA-treated embryos; - Development of pectoral fins of embryos was severely disturbed with exposure of thalomide, GA and TP; - Bradycardia was observed after exposure to MPA and thalidomide; - Pigmentation reduction after exposure to taxol, GA and TP; - Large yolk sac was observed after exposure to taxol, TP and MPA	(29)
Gambogic acid (GA)	0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8 $\mu mol.L^{-1}$				
Mycophenolic acid (MPA)	3.7, 11.1, 20, 33.3, 40, 50, 60, 75 $\mu mol.L^{-1}$				
Curcumin	10, 11, 12, 13, 14, 15, 16, 17, 18 $\mu mol.L^{-1}$				
Aurofin	1.56, 6.25, 10, 15, 20, 25 $\mu mol.L^{-1}$				
Thalomide	25, 25.5, 26, 26.5, 27, 27.5, 28, 28.5, 29, 29.5, 30 $\mu mol.L^{-1}$				
Taxol	0.5, 1, 2, 4, 6, 8, 10 $\mu mol.L^{-1}$				

4. Use and presence in the environment

The use of cytostatic drugs has increased due to the escalating incidence and early diagnosis of cancer. Data on patients with different type of cancers, including the most common, were collected by American Cancer Society between 2019 and 2021, which reports the treatment standards for these types of cancer and demonstrates the increased used of these compounds due to the great incidence of cancer in population. **Figure 3** shows the chemotherapy treatment rates, where cytostatic drugs are used, for the cancers selected in the different stages (30). As a result of the increase in its use, many parent compounds, metabolites and residues of cytostatic drugs, excreted the urine and feces, have been released in the environment leading to detection (17). Nowadays pharmaceuticals are recognized as important emerging environmental contaminants, as a result of inefficient wastewater treatment facilities in the removal and degradation of these hazardous compounds (10,22). Pharmaceutical residues found in the aquatic environment occur as mixtures, making the degradation of these compounds more difficult (2).

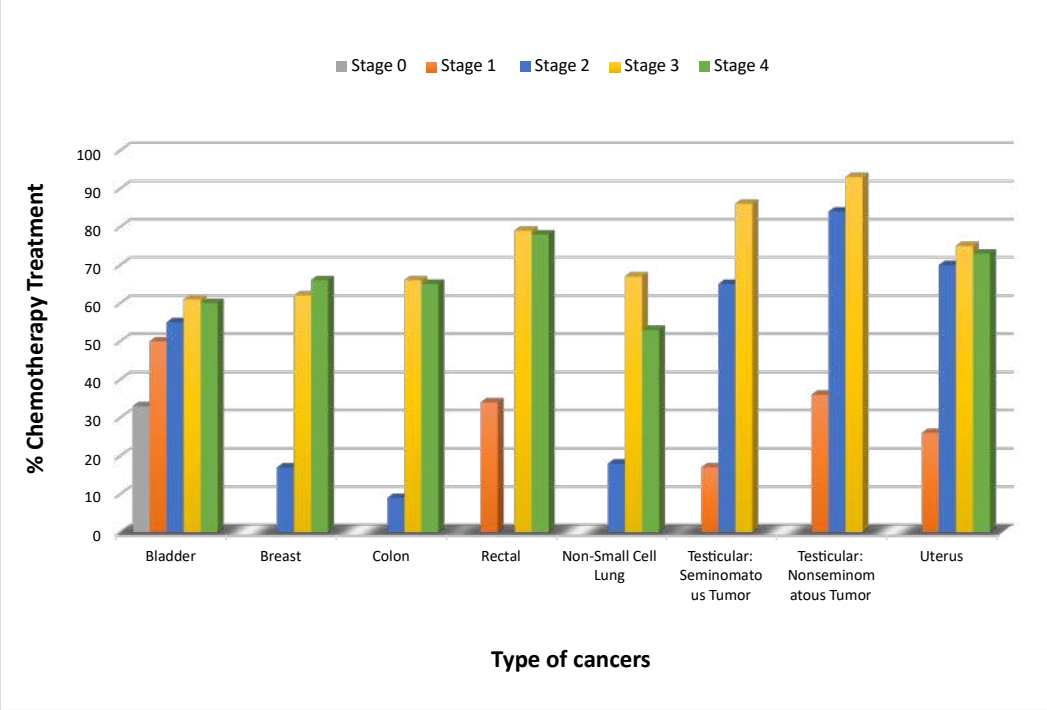

Figure 2. Chemotherapy treatment rates for selected cancers included in the American Cancer Society between 2019 and 2021 (Adapted from (30)).

Thus, cytostatic drugs, which have significant organ toxicity and are potentially carcinogenic to humans, are found in rivers and estuarine waters.

Hospital effluents account for 5.5% to 17% of the total discharged (10). The concentration of different drugs in WTTP effluents, WWTP influents, hospital effluents and river waters are shown in **Table 3**. Cytostatic drugs have low biodegradability when compared to other groups of pharmaceuticals, such as selective serotonin reputable inhibitors, and their degradation in surface water may be negligible (13,15,22,31).

The mode of action of anticancer drugs, made by influencing the stability of the genetic material by interfering with the function of DNA, a molecule common to all taxa, raises great concern with its presence in the environment. Furthermore, aquatic biota long-term exposure to these drugs can cause delayed toxic effects on aquatic life and, eventually effects on human health, representing a potentially serious environmental risk with unknown long-term effects, thus, raising concerns on potential effects on biodiversity preservation and environmental safety (2,10,32).

Table 3. Environmental levels of different cytostatic drugs in different countries.

Cytostatic drug	Country	Water type	Concentration range ($ng.L^{-1}$)	References
Bicalutamide	Spain	WWTP effluent	156	(13)
	Spain	Surface water	0.72-6.03	
	France		10.84	
	Spain		0.646	
	Japan	WWTP effluent	254	(11)
		Surface water	245	
Bleomycin	SE England	WWTP effluent	11-19	(2)
	France	WWTP influent	11-19	
		Hospital effluent	30-124	
	SE England	Surface water	5-17	
	German	Potable water	5-13	

Table 3 (Continuation). Environmental levels of different cytostatic drugs in different countries.

Cytostatic drug	Country	Water type	Concentration range ($ng.L^{-1}$)	References
Capecitabine	Spain	WWTP effluent	201	(13)
	United Kingdom		13.7-39	
	Czech Republic		87	
	Spain	WWTP influent	27	
	France	Surface water	3.52	
	United Kingdom		2.3	
	Spain		7.76	
	Spain	WWTP effluent	92	(33)
		Surface water	0.37-1.21	
Carboplatin	Iran	WWTP effluent	1200	(11)
		WWTP influent	>1600	
Cisplatin	Iran	WWTP effluent	430	(11)
		WWTP influent	>1120	
	France	Hospital effluent	1700-266000	
	Spain	Surface water	<56	
	France	WWTP effluent	0.4-0.5	(22)

Table 3 (Continuation). Environmental levels of different cytostatic drugs in different countries.

Cytostatic drug	Country	Water type	Concentration range ($ng.L^{-1}$)	References
Cyclophosphamide	Spain	WWTP influent	13100	(11)
	France	Hospital effluent	687000	
	Spain	WWTP effluent	2.94-43.5	(13)
	United Kingdom		70.2	
	Spain	Surface water	0.11-4.56	
	France		0.23-1.74	
	United Kingdom		4.1	
	Germany		0.19-0.60	
Docetaxel	France	WWTP effluent	Not detected	(11)
		WWTP influent	>219	
Epirubicin	Spain	WWTP effluent	24800	(11)
Erlotinib	Germany	WWTP effluent	0.9	(34)
		WWTP influent	0.5	
Etoposide	France	WWTP effluent	In 2004: 7 In 2008: 0.8	(22)
	North-Western England	WWTP effluent	1.3	
		Surface water	0.1	
	China	WWTP effluent	42	(11)
Exemestane	France	WWTP effluent	0.06	(13)

Table 3 (Continuation). Environmental levels of different cytostatic drugs in different countries.

Cytostatic drug	Country	Water type	Concentration range ($ng.L^{-1}$)	References
5-Fluorouracil	Spain	WWTP effluent	0.21	(11)
		WWTP influent	38	
	France	Hospital effluents	1280000	
	United Kingdom	WWTP effluent	23	(13)
	Spain	Surface water	7.91-44.1	
	United Kingdom		0.9	
	Germany	Hospital effluent	1280000	(35)
		Surface water	<0.01	
	Europe	Hospital effluent	23	(22)
		Municipal wastewaters	700	
	Germany	Surface water	<1	
Flutamide	Spain	WWTP effluent	18.8	(13)
	Spain	Surface water	0.25-0.73	
	France		0.16-1.19	
Gemcitabine	Spain	WWTP effluent	>50	(11)
Hydroxycarbamide	France	WWTP effluent	781	(13)
		Surface water	10.3	
	New England	WWTP effluent	781	(33)
		Surface water	0.13	

Table 3 (Continuation). Environmental levels of different cytostatic drugs in different countries.

Cytostatic drug	Country	Water type	Concentration range ($ng.L^{-1}$)	References
Ifosfamide	Spain	WWTP effluent	8.75-38	(13)
	France	Surface water	0.16-1.18	
	Germany		0.19-0.60	
	United Kingdom		0.1	
	Spain		0.34-3.80	
	France	Hospital effluent	6820000	(11)
Imatinib	Spain	WWTP influent	54-180	(11)
		WWTP effluent	60.6	(13)
	France	Surface water	4.99	
	United Kingdom		0.50	
	Spain	WWTP effluent	18.6	
		Surface water	0.209-6.16	
Methotrexate	Spain	WWTP effluent	66	(11)
		WWTP influent	308	
	France	Hospital effluent	3990	
	Germany	WWTP effluent	0.5	(34)
		WWTP influent	0.6	
Mycophenolic acid	Portugal	WWTP effluent	395-874	(11)
Oxaliplatin	France	WWTP effluent	<50	(11)
		WWTP influent	>600	

Table 3 (Continuation). Environmental levels of different cytostatic drugs in different countries.

Cytostatic drug	Country	Water type	Concentration range ($ng.L^{-1}$)	References
Tamoxifen	Spain	WWTP effluent	1.22	(13)
	Spain	Surface water	0.08-0.209	(13)
	France		1.14-8.61	
	England	WWTP effluent	369	(11)
		WWTP influent	215	
Tyrosine-kinase-inhibitors	France	WWTP effluent	13-20	(22)
Vincristine	China	WWTP effluent	Presence	(2)
		WWTP influent	22.9	
		Surface water	Presence	
	California	Hospital effluent	<20-50	

There are seven cytostatic drugs that are more likely to be found in surface an wastewater which are cyclophosphamide, capecitabine, mycophenolic acid, imatinib, bicalutamide, prednisone and 5-fluorouracil (32). However, as depicted in **Table 3**, cisplatin, methotrexate, gemcitabine, bicalutamide, cytarabine and ifosfamide were detected in surface waters posing a low environmental risk (concentration range ($ng.L^{-1}$): 0.4-38) while a moderate to high risk has been assigned to cyclophosphamide, mycophenolic acid, doxorubicin, fluorouracil, tamoxifen and vinorelbine (concentration range ($ng.L^{-1}$): 2.94-874) (11).

5. Conclusion and future trends

Cytostatics are increasingly used due to the rise of cancer cases, as a result of their application in chemotherapy. This usage causes an increase in the release of these drugs into the environment, which even at low concentrations has already proved capable of affecting

non-target organisms and even humans through drinking water, for example. Some cytostatic drugs, such as cyclophosphamide with WWTP influent values around 13100 $ng.L^{-1}$ and WWTP effluent values around 2.94-70.2 $ng.L^{-1}$, can be considered very dangerous for the environment. The environmental values of cytostatics such as cyclophosphamide, methotrexate and 5-fluorouracil, reflect the use of these drugs in cancer and other diseases, as they are three of the most used cytostatics and their values in the environment are higher than others that are not so used. Therefore, it would be important to better understand the pathways of pharmaceutical pollution in the environment, in order to try to decrease this pollution and its effects on non-target organisms.

In the future, it would be important and essential to invest in studies with cytostatic drugs, in order to better understand the effects that these drugs can have on non-target organisms, individually and when combined. It would also be important to try to improve the efficiency of wastewater treatment plants and/or to find cytostatics that cause less effects on non-target organisms.

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Chapter III

Effects of Methotrexate, 5-Fluorouracil and this mixture on zebrafish embryos

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Abstract

Methotrexate (MTX) and 5-Fluorouracil (5-FU) are two of the mostly used cytostatics in the treatment of cancer and, consequently, more present in the environment. In order to have a better understanding of the mechanisms and effects of cytostatics on non-target organisms, physiological, biochemical and behavioral endpoints were evaluated on zebrafish embryos, in single and combined exposures. All tests performed showed differences in all parameters evaluated (toxicity, behavior and heartbeat rate). With methotrexate these differences were found predominantly in the highest concentration (1000 $\mu g.L^{-1}$), while in the remaining tests there are differences in almost all concentrations tested. In all the tests, the drugs were shown to affect mainly the tail and the heart. Both caused stress in the zebrafish, however 5-FU appears to be more toxic than MTX, since it caused more side effects.

Keywords: Methotrexate, 5-Fluorouracil, FET, Biochemical endpoints, Development endpoints, Synergic mixture

1. Introduction

The use of cytostatic drugs has increased due to the high incidence of cancer in the population worldwide (1). Methotrexate (MTX) and 5-Fluorouracil (5-FU) are two of the mostly used cytostatics in chemotherapy in the world (2). Both drugs can raise toxic effects on the environment and in human health, such as genotoxic, mutagenic and teratogenic effects. However, these toxic effects can be more serious if they are complex drug mixtures (1). In this work two cytostatic drugs, MTX and 5-FU, and their mixture were analyzed.

1.1. Methotrexate

Methotrexate (MTX) or 4-amino-N^{10}-methypteroglutamic acid with the chemical formula $C_{20}H_{22}N_8O_5$ (**Figure 1**) and the molecular weight of 454.45 $g.mol^{-1}$, is the first antagonist of folic acid developed for the treatment of malignancies (2). It is used to treat different diseases such as cancer, ectopic pregnancy and autoimmune disorders (3).

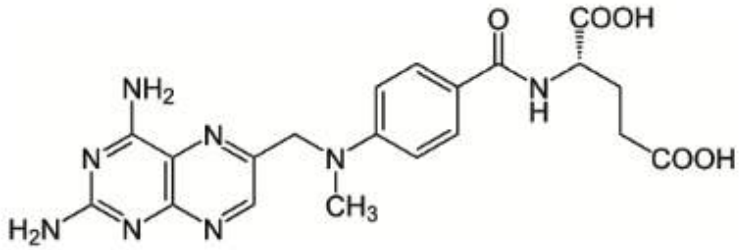


Figure 1. Chemical structure of methotrexate (4).

The most common types of cancer treated with MTX are leukemia and the cancers of the breast, skin, head and neck, lung, or uterus (5). Rheumatoid arthritis, an autoimmune, chronic, symmetrical and inflammatory disease, and psoriasis, a chronic inflammatory and common skin disease, are autoimmune disorders treated with MTX once this drug has immunosuppressive, cytostatic and anti-inflammatory effects (2,6,7). It is also used in ectopic pregnancy, characterized by an implantation of fertilized oocyte outside the uterine cavity (8).

Methotrexate remains as a first-line drug due to its cost-effectiveness, despite its potential side effects. Close monitoring of total blood count and liver function makes long-term administration in psoriasis feasible (7).

MTX can be given in low or high doses, depending on the treatment. If the treatment is chemotherapy, cancer in initial conditions, rheumatic diseases or psoriasis, the applied dose is low (e.g. 20 mg/m^2) and normally is safe and well tolerated. In other cases, such as the treatment of certain cancers such as primary central nervous system lymphoma, MTX can be given in higher doses (e.g. 1000 mg/m^2 – 33000 mg/m^2). However, higher doses of MTX therapy can cause significant toxicity like nephrotoxicity, myelosuppression, mucositis, hepatoxicity, among other toxic effects (2). It may cause multi-organ failure in

severe cases (2). This drug is eliminated by renal excretion involving passive glomerular filtration and active tubular reabsorption and secretion (9). If methotrexate is administered intravenously, 80-90% of the administered dose is excreted unchanged in the urine within 24h. After excretion, it enters the environment via hospital wastewaters, urban wastewaters and can be detected even in drinking water and can affect non-target aquatic animals and humans (9).

Methotrexate is a hydrophilic molecule and can only enter the cell through active transport process. MTX inhibits the synthesis of DNA, RNA, thymidylates, and proteins. After crossing the cell membrane, this molecule is converted to a polyglutamate form (**Figure 2**), which prevents the transport of methotrexate outside the cells (10).

Methotrexate is an antagonist of folic acid, an important DNA precursor (2). Folic acid undergoes reduction to dihydrofolate and then tetrahydrofolate by the enzyme dihydrofolate reductase (DHFR), which acts as a cofactor for thymidylate synthase (TS) that catalyzes the synthesis of pyrimidine converting deoxyuridine monophosphate (dUMP) to deoxythymidine monophosphate (dTMP) (**Figure 2**) (2). It is thus needed for the synthesis of the nucleoside, thymidine, required for DNA synthesis (10). Methotrexate has more affinity with dihydrofolate reductase than folate and competitively and irreversibly inhibits DHFR, reducing folate pool (2,10). The effectiveness of methotrexate increases when larger and more hydrophilic metabolites are retained by the cell. The affinity of methotrexate for other target enzymes (thymidylate synthetase) and enzymes of the purine synthesis pathway also increases when glutamate residues are added to methotrexate (10).

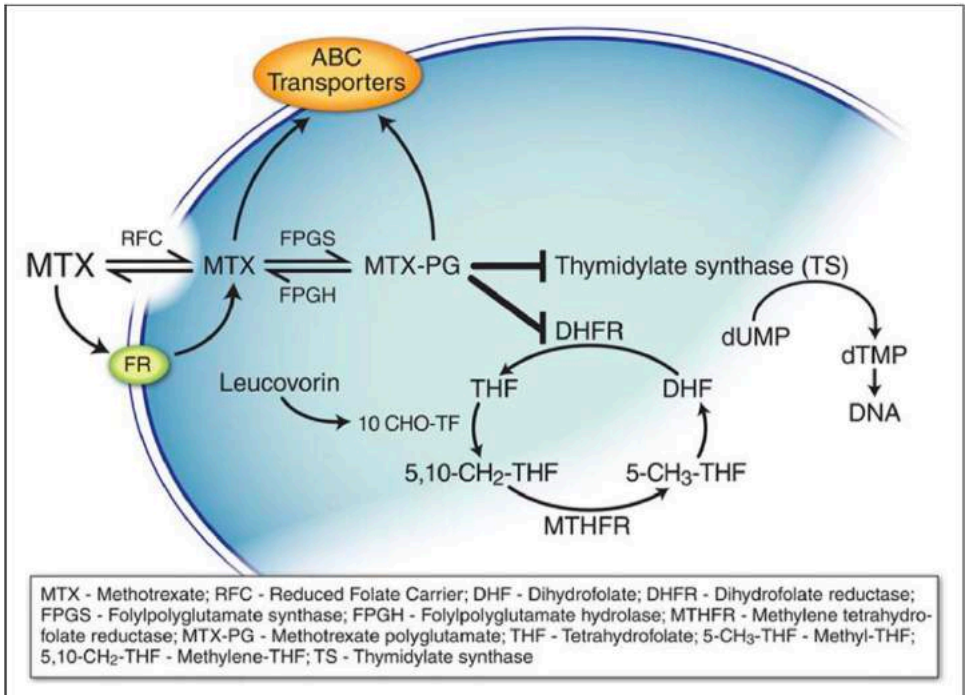


Figure 2. Mechanism of action of methotrexate (from (10)).

1.2. 5-Fluorouracil

5-Fluorouracil (5-FU) with the chemical formula $C_4H_3FN_2O_2$ (**Figure 3**) and with a molecular weight of 130.08 $g.mol^{-1}$, is the most widely used active antineoplastic agent in the world (11). It is a fluorinated pyrimidine analogue in the treatment of cancer, which acts as an antimetabolite (12). 5-FU is a dipodic acid with high polarity and has a low molecular weight (2).

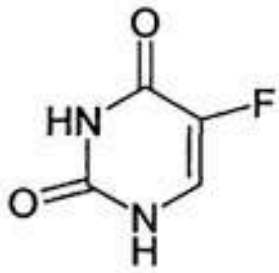


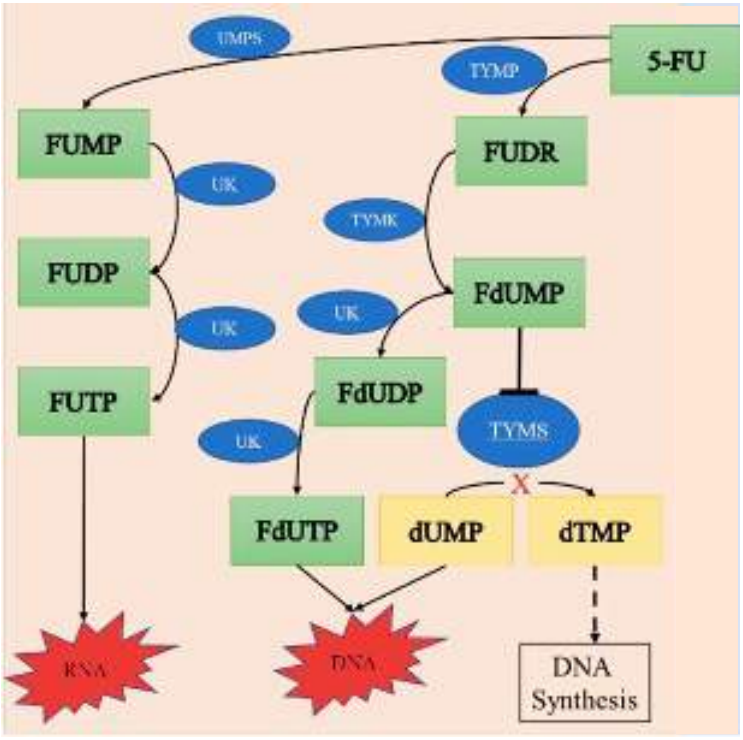
Figure 3. Chemical structure of 5-fluorouracil (13).

5-FU is used in chemotherapy for local and systemic cancer treatment (e.g. solid tumors such as colorectal, breast, aerodigestive tract, head and neck cancers), as well as several structurally related derivatives like capecitabine (CAP), which is a prodrug of 5-FU (2,11,12). CAP is a fluoropyrimidine carbamate and it is metabolized to the active substance 5-FU in the body more in tumor cells than in normal tissues (12).

This drug can have some common side effects like stomatitis, leukopenia, alopecia, nausea, vomiting, diarrhea, and significant side effects include gastrointestinal toxicity, bone marrow depression and cardiac toxicity (2,11). However, there is a high interpatient and intrapatient pharmacokinetic variability resulting in an inadequate dosage (11).

In the water environment samples, 5-FU can be efficiently removed by photolysis, leading to a low concentration in the environment, 0.21 ngL^{-1} in the effluent and < 0.01 ngL^{-1} in the influent. However, even at low concentrations 5-FU can affect non-target animals. Histopathological changes in the liver and kidneys and genotoxic effects have been reported in zebrafish exposed to 10 ngL^{-1}, for 33 days in a sub-chronic toxicity assay. This suggests that other non-target organisms in the environment may be susceptible to their presence in environment (12).

The main mechanism of action of 5-FU involves the inhibition of thymidylate synthase (TYMS) and the incorporation of 5-FU in RNA and DNA (**Figure 4**) (2). 5-FU is converted to fluorouridine monophosphate (FUMP) by uridine monophosphate synthetase (UMPS) and phosphorylated by uridine kinase (UK) (14). After FUMP is phosphorylated to fluorouridine diphosphate (FUDP), it can be phosphorylated to fluorouridine triphosphate (FUTP). FUTP is incorporated into the RNA and can be disrupt its functions (2). An alternative activation pathway is converting 5-FU to fluorouridine (FUDR) catalyzed by thymidine phosphorylase (TYMP), which is then phosphorylated by thymidylate kinase (TYMK) to fluorodeoxyuridine monophosphate (FdUMP) (14). Thymidylate synthase (TYMS) is inhibited by FdUMP causing an imbalance of deoxythymidine monophosphate (dTMP) and deoxyuridine monophosphate (dUMP). Incorporation of dUMP and FdUTP into DNA causes damage and leads to cell death (14).



TYMP- thymidine phosphorylase
UMPS- uridine monophosphate synthetase
UK- uridine kinase
TYMK- thymidylate kinase
TYMS- thymidylate synthase
FUMP- fluorouridine monophosphate
FUDP- fluorouridine diphosphate
FUTP- fluorouridine triphosphate
FUDR- fluorouridine
FdUMP- fluorodeoxyuridine monophosphate
FdUTP- fluorodeoxyuridine triphosphate
dUMP- deoxyuridine monophosphate
dTMP- deoxythymidine monophosphate

Figure 4. Mechanism of action of 5-fluorouracil (adapted from (14)).

The aim of this work is to evaluate the physiological and biochemical as well as behavioral responses provoked by two cytostatic compounds, methotrexate and 5-fluorouracil, individually and in combination, in zebrafish, after short-exposure (96h), and in a range of concentrations, including environmentally relevant concentrations.

2. Material and Methods

2.1. Test organisms

Embryos of wild type zebrafish (*Danio rerio*) were used as a biological model and supplied by the laboratory culture kept at the Department of Biology (University of Aveiro, Portugal). Organisms kept in a recirculating system (ZebTEC; Tecniplast) with reverse osmosis and tap water filtered with activated carbon, complemented with salt "Instant Ocean Synthetic Sea Salt" (Spectrum Brands, USA) (automatically adjusted for pH and conductivity) were used as a source of embryos for the experimental assays. Animals were

maintained in aquaria with water temperature of 26.0 ± 1 ºC, conductivity 750 ± 50 mS/cm, pH 7.5 ± 0.5, salinity of 0.35 ppt and dissolved oxygen equal to or greater than ≥ 95% saturation at a 16:8 h (light: dark) photoperiod cycle. The adult fish were fed daily with a commercial artificial diet (ZM-400 fish food; Zebrafish Management Ltd).

Zebrafish eggs used for this study, were collected within 30 min after natural mating, rinsed in fish culture water, and screened using a stereomicroscope (Stereoscopic Zoom Microscope-SMZ 1500, Nikon Corporation) to exclude unfertilized eggs, injured or with irregular cleavage.

2.2. Test chemicals

Methotrexate (4-amino-N^{10}-methypteroglutamic acid) and 5-Fluorouracil were acquired from Tokyo Chemical Industry, Belgium. All other reagents were analytical grade and were acquired from Sigma Aldrich (Spain).

2.3. Exposure conditions

Zebrafish embryos were exposed to several concentrations of methotrexate, 5-fluorouracil and this mixture. Four exposures were made: 1) Methotrexate (0.01, 0.1, 1, 10, 100 and 1000 $\mu g.L^{-1}$) performed in light; 2) Methotrexate (0.01, 0.1, 1, 10, 100 and 1000 $\mu g.L^{-1}$) performed in dark; 3) 5-Fluorouracil (0.01, 0.1, 1, 10, 100 and 1000 $\mu g.L^{-1}$); 4) Mixture of methotrexate and 5-fluorouracil (10, 100 and 1000 $\mu g.L^{-1}$). Two exposures were made with methotrexate, which is a photosensitive drug and can be degraded in the presence of light. To avoid less reliable results due to this factor, a second test was performed in the absence of light. Stock solutions were prepared by dissolution in fish culture water (water from the recirculating system).

Two different sets of experiments were performed in this study to assess effects on development, behavior and biochemical endpoints. In all assays, the newly fertilized eggs (3h post-fertilization- hpf) were kept in the dark, under controlled temperature (27 ± 1 ºC) to avoid photodegradation of methotrexate.

2.4. Fish embryo toxicity (FET) assay

The experimental design was based on the OECD testing guideline 236 for fish embryo toxicity (FET) test (**OECD, 2013**). The design of the lethal assay included concentrations of methotrexate, 5-fluorouracil and mixture of methotrexate and 5-fluorouracil and also a negative control (water system only). A total of 20 embryos per treatment were distributed individually in 24-well plates, containing 2 mL of test solution, with four plate control organisms. Test run for 96 h under controlled condition (27 ± 1 ºC, 16:8h (light:dark) photoperiod cycle). Embryos were observed daily under a stereomicroscope and mortality and deformations were recorded.

Some parameters were evaluated to determine the effects of methotrexate, 5-fluorouracil and of a mixture of methotrexate and 5-fluorouracil on the development of fish embryos: heartbeat (at 48 hpf), hatching (at 48 hpf), and larval malformations up to 96 hpf. All parameters were evaluated qualitatively. The heartbeat (beats/min) was assessed by counting heartbeats under a stereomicroscope in 10 embryos randomly selected from each replica and during 15 s (n= 8 per concentration).

2.5. Behavior assay

For the behavior assay, a total of 20 embryos were individually exposed in 24-well plates per treatment, using a random design for the distribution of treatments on the plate, to avoid bias. Locomotion was evaluated at 120 hpf to ensure that all larvae had an inflated swimming bladder, allowing free swimming. Dead larvae or larvae with physical abnormalities were excluded. At this stage of development, larvae still feed on their yolk sac. Locomotor activity was measured using a ZebraBox- ZEB 478 (Viewpoint Life sciences, Lyon, France), automated video recording equipped with internal LED lights over a period of 6 min. This system monitors movement by automated video recording using a tracking setting. Zebrafish larvae were subjected to one cycle of light and dark, 3 minutes each. Inactivity/slow movements, medium movements, rapid movements, and the distance and time were evaluated. For background correction, a threshold of 30 has been set. The total distance moved (TD, overall distance moved during a defined period of time, in mm)

was recorded. Moreover, three types of movements were considered: low velocity (hypoactivity and inactivity) < 8 mm/s; medium velocity for movements between 8 and 40 mm/s (normal activity) and high velocity (hyperactivity) >40 mm/s. Percentage of time or distance moved in each type of movement was calculated dividing the respective time or distance by the total swimming time or distance and multiplying by 100.

In the protocol built on the ZebraLab® v3 Automated Behavioral Analysis program, two monitoring zones were defined in the recording area: an inner and an outer zone, allowing the analysis of the tendency to swim near the edges of the container (as a measure of thigmotatic behavior). Given that distance swum, to analyze tendency to swim near the edges the percentage of distance swum in this zone was calculated (dividing the distance moved in the outer area by the overall swimming distance and multiplying by 100).

2.6. Biochemical assay

For the biochemical analysis, 10 embryos per replicate were pooled together without solution and stored at -80ºC until further processing. On the day of the analyses, samples were defrosted on ice, homogenized in potassium phosphate buffer (0.1M, pH 7.4) using a sonicator (Branson S-250A). Aliquots for post-mitochondrial supernatant (PMS) and Acetylcholinesterase (AChE) fraction isolation were sampled, were centrifuged (4ºC, 10000g, 20 min for PMS and 4ºC, 300g, 5 min for AChE) to determine the enzymatic activity.

Enzymatic determinations were made spectrophotometrically (Thermo Scientific Multiskan Spectrum), in triplicate, using a 96-well microplate and expressed in nano or micromoles of hydrolyzed substrate per minute per mg of protein. The protein concentrations in the fractions were determined based on the Bradford method (1976), using a wavelength of 595 nm and γ-globulin as standard. AChE activity was determined according to the method of Ellman (Ellman et al, 1961), using acetylthiocholine as substrate. The absorbable increase caused by the conjugation product of thiocoline (product of acetylthiocholine degradation) and 5,5-dithiobis-2-nitrobenzoic acid (DTNB) in phosphate-buffered saline (PBS) was measured at 414 nm for 5 min. Lactate dehydrogenase (LDH) activity was measured at 340 nm, following the Vassault method (Vassault, 1983) by

monitoring, for 5 min, the decrease in absorbance due to the oxidation of NADH. Glutathione S-Transferase (GST) activity was determined at 340 nm, as described by Habig and Jakob (1981), by monitoring, during 5 min, the increase in absorbance resulting from the conjugation product between reduced glutathione and 1-chloro-2,4-dinitrobenzene. Catalase (CAT) activity was measured at 240 nm as described by Claiborne (1985), monitoring the decrease in absorbance due to the degradation of hydrogen peroxide, for 2 min.

2.7. Data analysis

The SPSS 27.5. statistical package (SPSS Statistics) was used for all statistical analyses. Data on fish embryo toxicity, feeding behavior, development and biochemical endpoints were analyzed for normality and homogeneity. When normality and homogeneity of data were verified, one-way ANOVA was performed followed by the multiple comparison Dunnett's test to assess differences in relation to the towards control; otherwise, the non-parametric Krushkall-Wallis was done followed by the multiple comparison Dunn's test. All statistical analyses were performed with a significance level of 0.05.

3. Results of Methotrexate analysis

Two assays were performed with methotrexate, one in the light and one in the dark. Since this drug is considered photosensitive and the first assay was carried out in the light, we decided to repeat the assay but this time in the dark, in order to make the assay more reliable.

3.1. Heartbeat rate

The analysis of the cardiac activity (beats/min) at 48 hpf revealed no significant differences, in the first test, between groups in terms of heartbeat (**Figure 5, A**). However,

the results demonstrate a tendency for the heartbeat rate to be higher, the higher the concentration. The number of beats per min in all tested conditions varied between 140 and 196 ($p<0.05$). In the second test, significant differences were observed in the concentrations 0.01 $\mu g.L^{-1}$ and 1000 $\mu g.L^{-1}$, where in 0.01 $\mu g.L^{-1}$ the heartbeat rate was lower than the control and in 1000 $\mu g.L^{-1}$ the values were higher than the control (**Figure 5, B**). The number of beats per minute in all tested conditions varied between 68 and 148 ($p<0.05$).

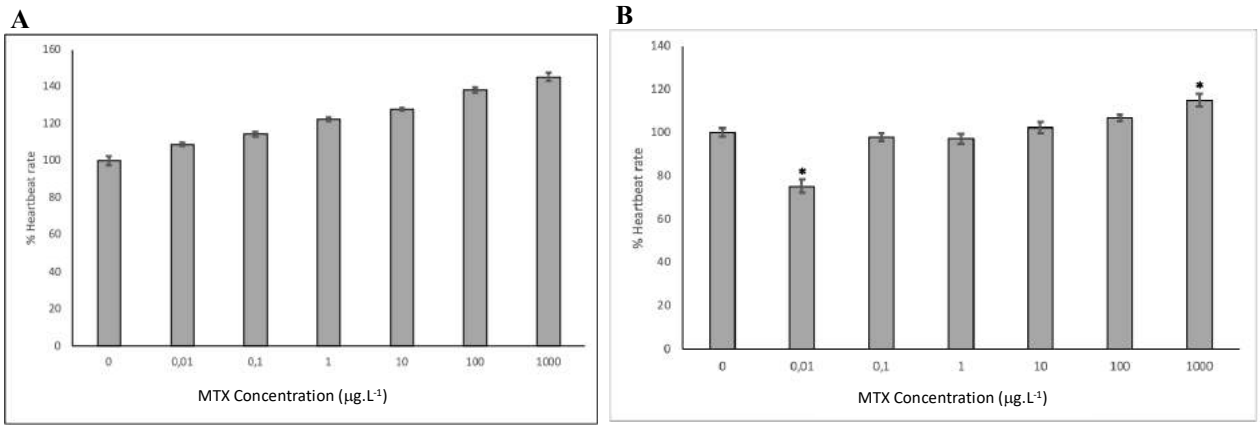


Figure 5. Heart beats measured at 48 hpf in zebrafish larvae after exposure to methotrexate. Values are expressed in beats per minute. **A-** First test; **B-** Second test. (*$p<0.05$)

3.2. Fish Embryo Toxicity (FET) assay

In this test some parameters were evaluated, however in the first test with MTX, it was only possible to observe significant differences in the following four parameters, transparent tail of the zebrafish, spontaneous movement, fish pigmentation and the number of live larvae. After 24 hours of testing, significant differences were observed in the transparent tail at concentrations 10, 100 and 1000 $\mu g.L^{-1}$ (**Figure 6, A**), since the larvae had a more transparent tail at these concentrations compared to the control. In the spontaneous movement, significant differences were observed in the concentration of 10 $\mu g.L^{-1}$ (**Figure 6, B**), where the larvae showed a spontaneous movement superior to the control.

After 48 hours of testing, significant differences were observed in fish pigmentation at concentrations of 0.01, 0.1 and 10 $\mu g.L^{-1}$ (**Figure 6, C**), where the larvae were more

pigmented at these concentrations than in control. In the number of live larvae, there were significant differences in concentrations of 10 and 100 μg.L^{-1} (**Figure 6, D**), with less live larvae in these concentrations than in control.

Although no significant differences were observed in other parameters like deformation, tail deformation or zebrafish's edema, **Figure 7** displays some zebrafish pictures taken during the test, where these alterations are visible.

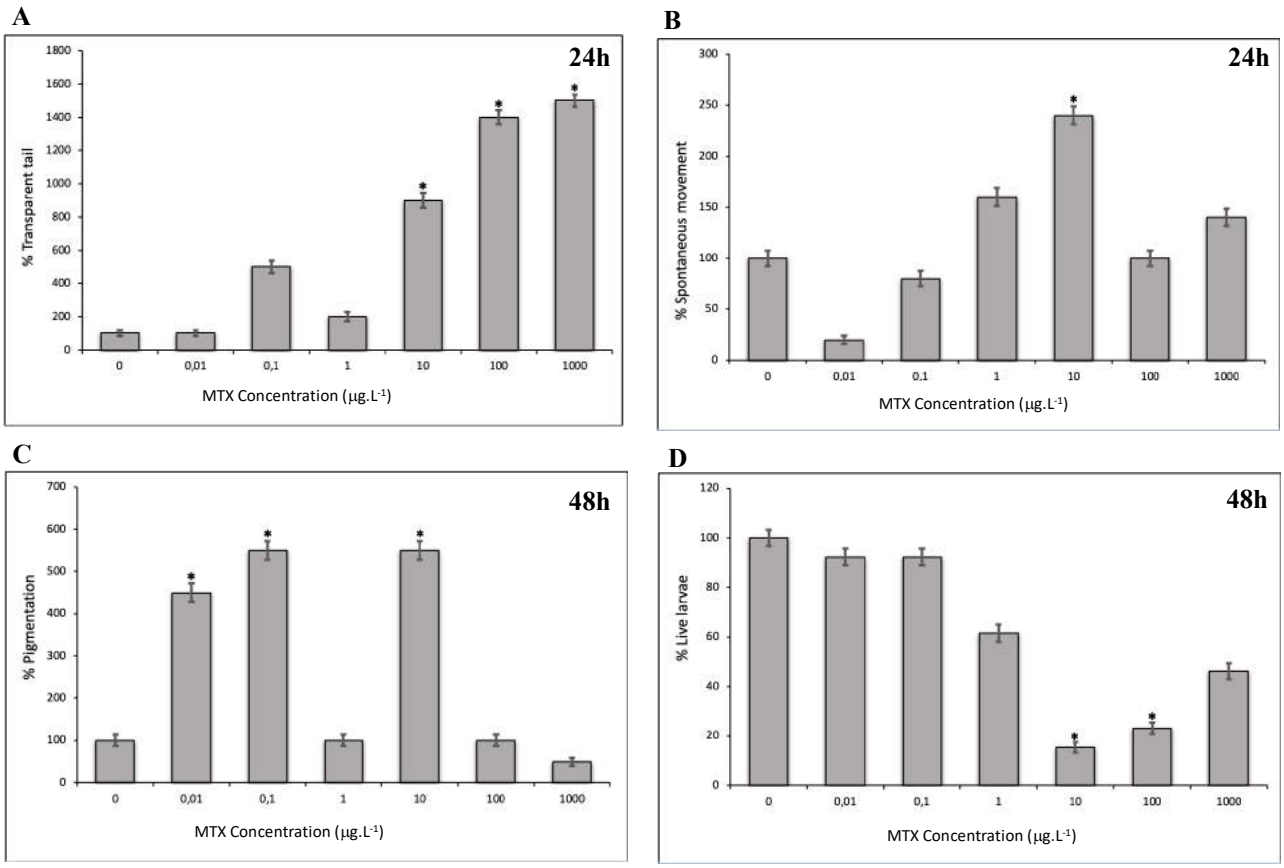


Figure 6. Evaluation of different parameters in zebrafish embryos after 24 and 48 hours of first exposure to methotrexate. **A-** Tail transparency; **B-** Spontaneous movement; **C-** Zebrafish Pigmentation; **D-** Live larvae. (*p<0.05)

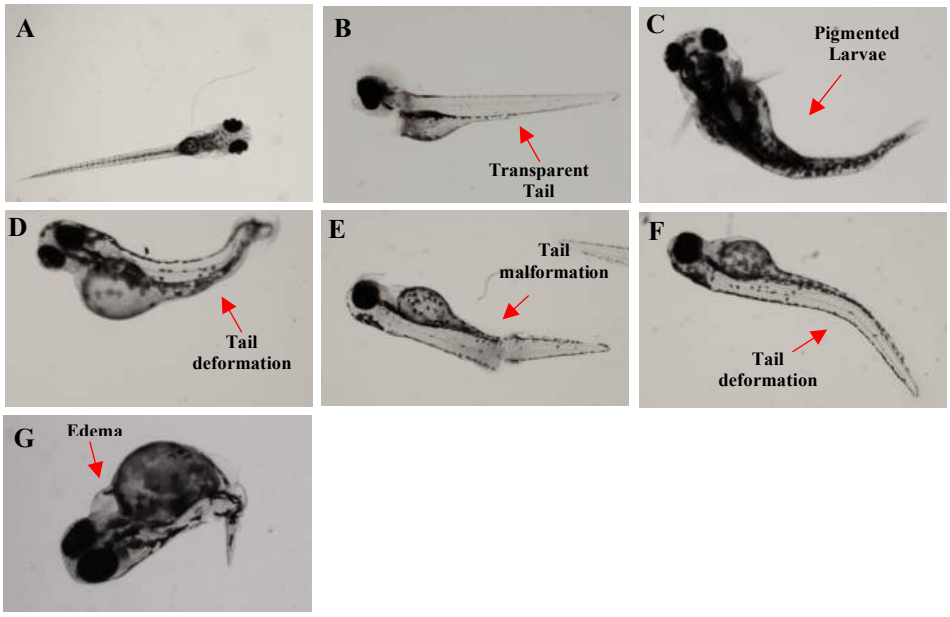
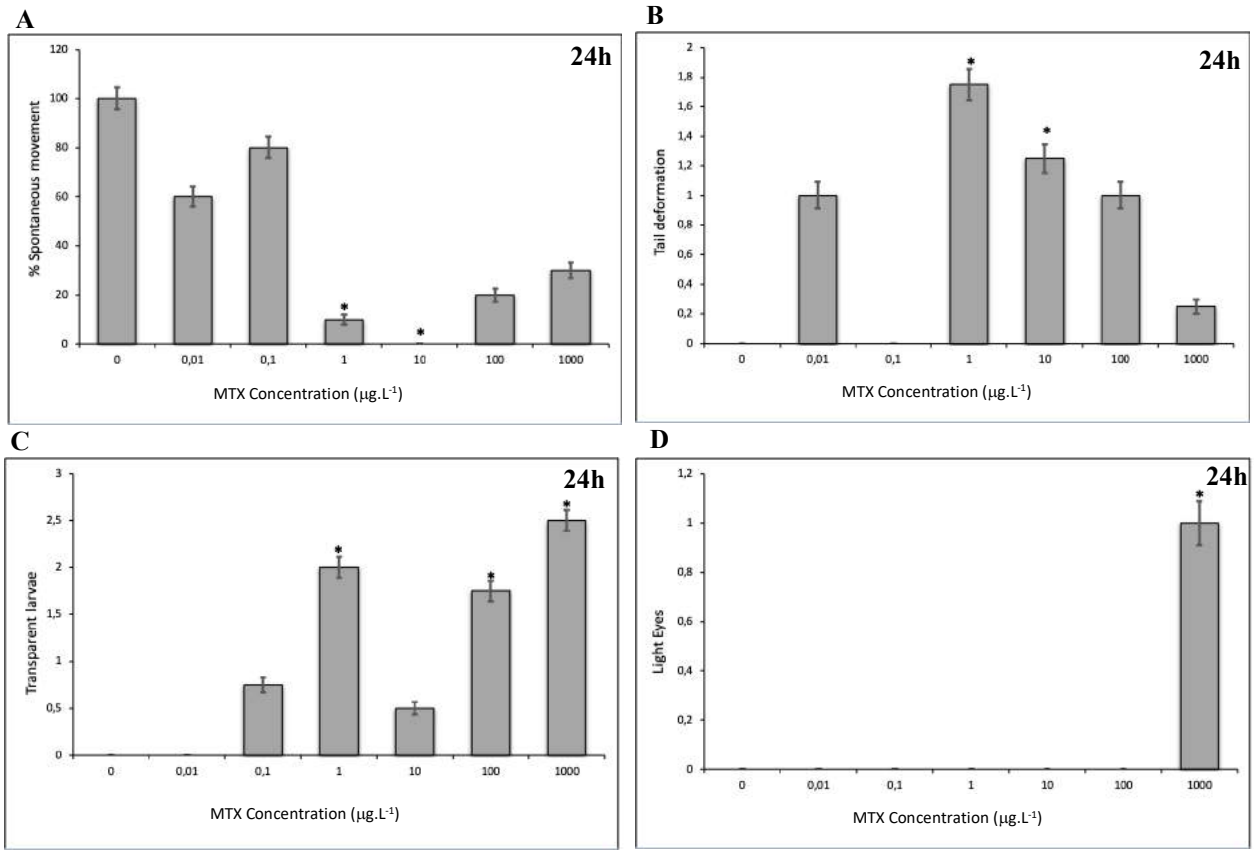


Figure 7. Representative specimens of zebrafish pictures after first exposure to methotrexate, with 100x ampliation, taken during the FET test. **A-** Control; **B-** Malformation ([MTX]= 0.1 $\mu g.L^{-1}$); **C-** Edema, Tail deformation and Pigmentation ([MTX]= 0.1 $\mu g.L^{-1}$); **D-** Tail deformation ([MTX]= 0.1 $\mu g.L^{-1}$); **E-** Tail malformation ([MTX]= 10 $\mu g.L^{-1}$); **F-** Tail deformation ([MTX]= 10 $\mu g.L^{-1}$); **G–** Edema ([MTX]= 1000 $\mu g.L^{-1}$).

In the second test with MTX, significant differences in parameters were observed, such as in spontaneous movement, tail deformation, transparent larvae, light eyes and larvae deformation. After 24 hours of testing, significant differences were observed in spontaneous movement at concentrations 1 and 10 $\mu g.L^{-1}$ (**Figure 8, A**), where the larvae showed a lower spontaneous movement at concentration 1 $\mu g.L^{-1}$ and had no movement at concentration 10 $\mu g.L^{-1}$ in relation to the control. Regarding tail deformation, in all concentrations with the exception 0.1 $\mu g.L^{-1}$ there was deformation, but more significant differences were observed in the concentrations of 1 and 10 $\mu g.L^{-1}$ (**Figure 8, B**) when compared to the control, which presented no tail deformation. Regarding larvae transparency (**Figure 8, C**), differences were observed in all concentrations except in the concentrations of 0.01 and 10 $\mu g.L^{-1}$, when compared to the control. Larvae with light eyes were observed only in the highest

concentration tested (1000 μg.L^{-1}) (**Figure 8, D**), and thus, there were only significant differences in this concentration in relation to the control. Lastly, after 96 hours of exposure, significant differences in terms of larvae deformation were only observed in a concentration of 100 μg.L^{-1} (**Figure 8, E**), which presented a higher number of deformations compared to the control.

In **Figure 9** are shown some pictures of the zebrafish taken during the test, where some alterations mentioned above are visible.



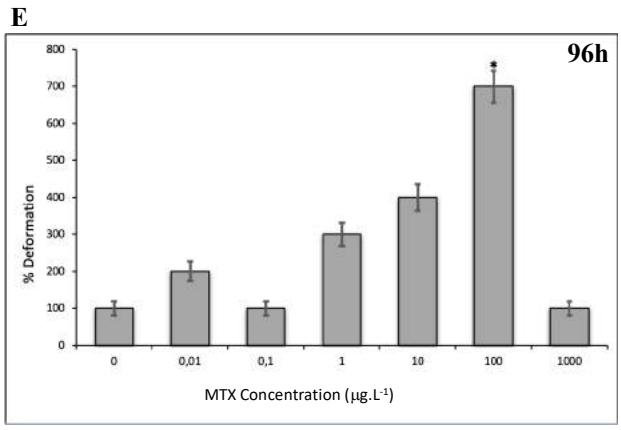


Figure 8. Evaluation of different parameters in zebrafish embryos after 24 and 96 hours of second exposure to methotrexate. **A-** Spontaneous movement; **B-** Tail transparent; **C-** Larvae transparent; **D-** Light eyes; **E-** Zebrafish deformation. (*p<0.05)

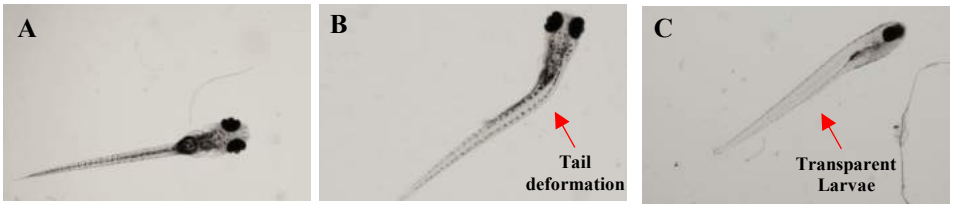


Figure 9. Representative specimens of zebrafish pictures after second exposure to methotrexate, with 100x ampliation, taken during the FET test. **A-** Control; **B-** Tail deformation ([MTX]= 0.01 µg.L^{-1}); **C-** Larvae transparent ([MTX]= 0.1 µg.L^{-1}).

3.3. Behavior assay

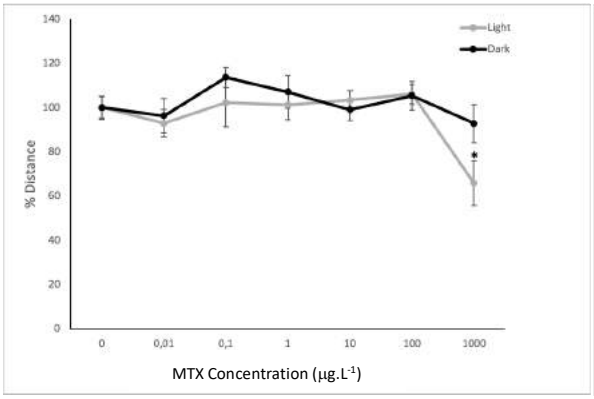
Fish exposed to methotrexate while in embryonic stage were subjected to 1 cycle of light and dark periods to assess locomotor activity and thigmotaxis. The reaction to the sudden changes in light conditions seemed to depend on the methotrexate concentration to which fish were exposed, with effects most evident at the highest concentration (1000 µg.L^{-1}) tested.

In the first test, significant differences were observed in all phases. In inactivity/slow movements (**Figure 10, A**), it is possible to observe significant differences in the light phase in the highest concentration (1000 μg.L^{-1}) compared to the control, both in distance and time. In this concentration, the fish traveled a shorter distance and the time was also shorter than those exposed to control. There were no effects of the remaining concentrations. No effects were found in the dark phase. In terms of medium movements (**Figure 10, B**), the response patterns were similar, with significant differences only found in the highest concentration (1000 μg.L^{-1}) in the light phase and no effects during the dark period. In terms of rapid movements (**Figure 10, C**), significant differences at concentration 1 μg.L^{-1} in both distance and time was observed. This difference occurs in the light phase in the distance (**Figure 10, C1**), but in time (**Figure 10, C2**) it occurs in both phases, with a higher value compared to the control. In total distance and in total time (**Figure 10, D**), the same can be observed in Figure 10A and Figure 10B, only with significant differences in the highest concentration (1000 μg.L^{-1}) tested, where the values were lower than in the control. In distance out and in time out (**Figure 10, E**), it is the same situation, however the values in 1000 μg.L^{-1} were higher than in the control and in the other concentrations.

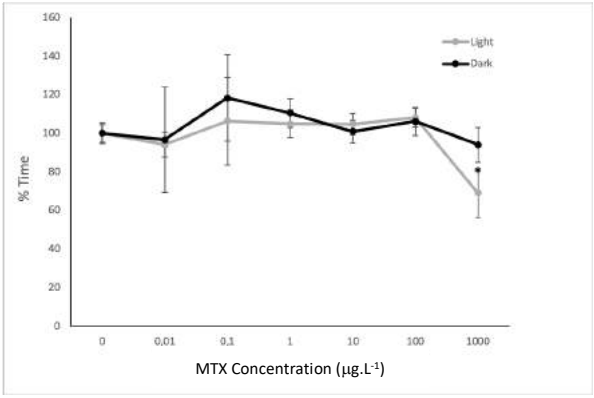
In general, embryos performance is better in the dark phase than in the light phase.

A. Inactivity/slow movements

1

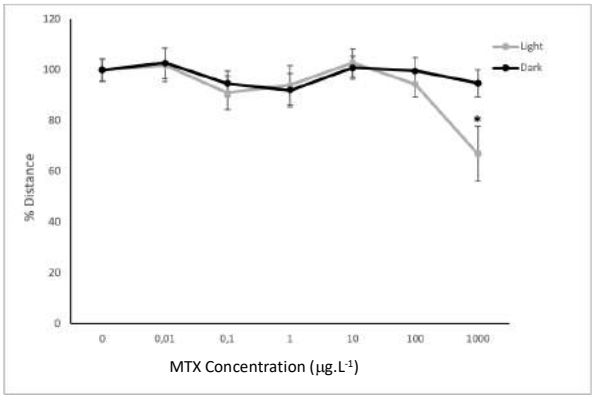


2



B. Medium movements

1



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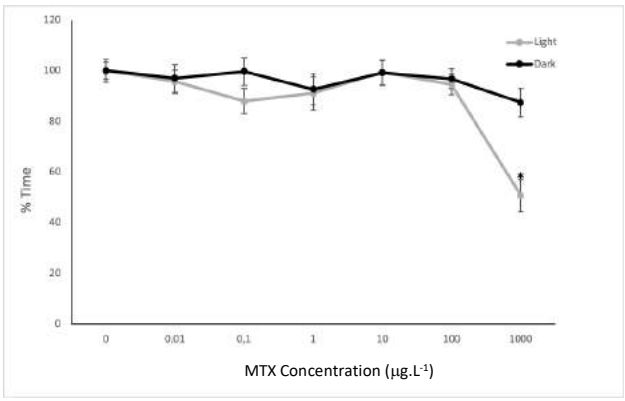
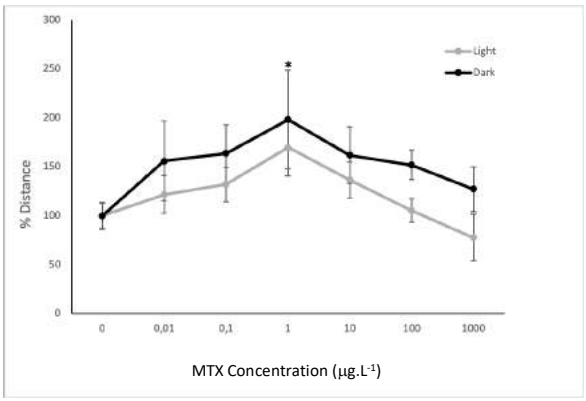


C. Rapid movements

1



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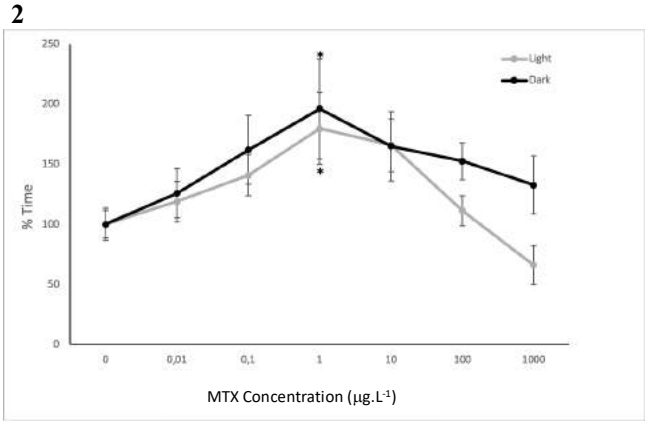
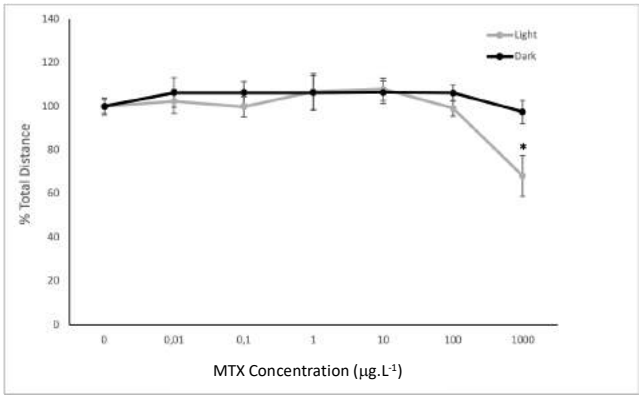


D. Total distance and total time

1



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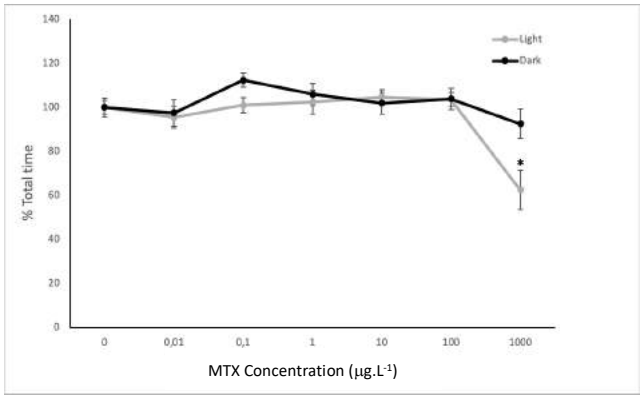


E. Distance out and time out

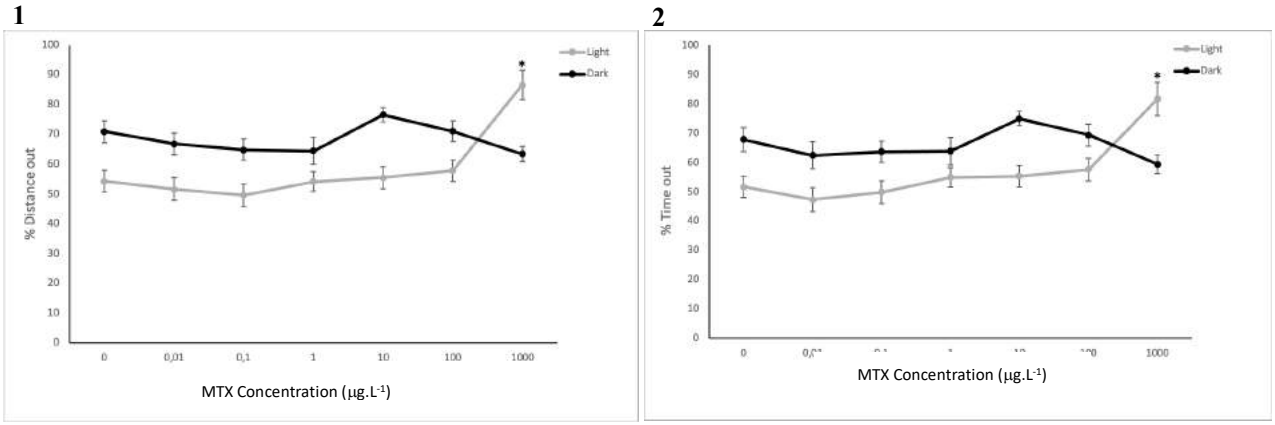


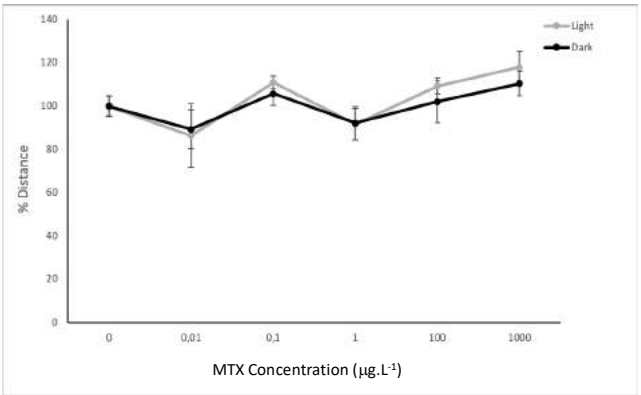
Figure 10. Evaluation of locomotor activity and thigmotaxis of zebrafish after first exposure to methotrexate. **A-** Inactivity/slow movements (A1- Distance; A2- Time); **B-** Medium movements (B1- Distance; B2- Time); **C-** Rapid movements (C1-Distance; C2- Time); **D-** Total distance and total time (D1- Total distance; D2- Total time); **E-** Distance out and time out (E1- Distance out; E2- Time out). (*p<0.05)

In the second test, in inactivity/slow movements (**Figure 11, A**), significant differences in the light phase at the highest concentration (1000 µg.L^{-1}) in relation to the control were observed, only in time (**Figure 11, A2**). At this concentration, the fish is slower than the control. No significant differences were observed in terms of distance swam (**Figure 11, A1**). In terms of medium movements (**Figure 11, B**), significant differences were observed in distance and time. Concerning distance (**Figure 11, B1**), there were differences in the concentrations of 100 and 1000 µg.L^{-1} in the light phase, with the values being lower than the control. Concerning time (**Figure 11, B2**), there were only differences in 1000 µg.L^{-1} in the light phase and the values are also lower than the control. In rapid movements (**Figure 11, C**), it is possible to observe significant differences in the concentration 1000 µg.L^{-1} in the distance (**Figure 11, C1**) and 100 and 1000 µg.L^{-1} in time (**Figure 11, C2**). In the distance, this difference occurs in the light phase, with a lower value compared to the control. However, over time, it occurs in both phases, at 100 µg.L^{-1} in the dark phase and at 1000 µg.L^{-1} in the light phase, with a lower value than the control in both concentrations. In the total distance and in total time (**Figure 11, D**), significant differences were observed only in the distance (**Figure 11, D1**) at 1000 µg.L^{-1} in light phase, with the value being lower

than the control. No significant differences were observed over time. In the distance out and in time out (**Figure 11, E**), no significant differences were observed neither in distance nor time.

A. Inactivity/slow movements

1



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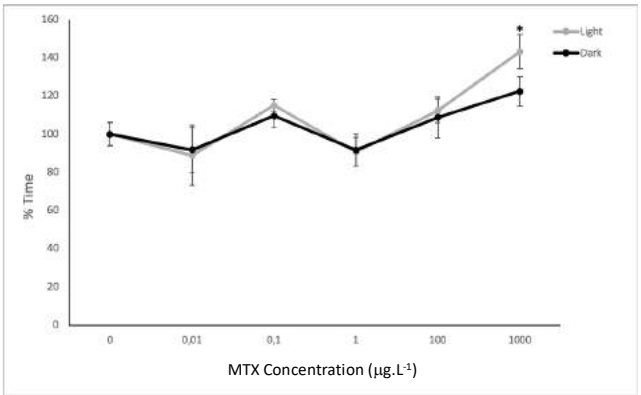
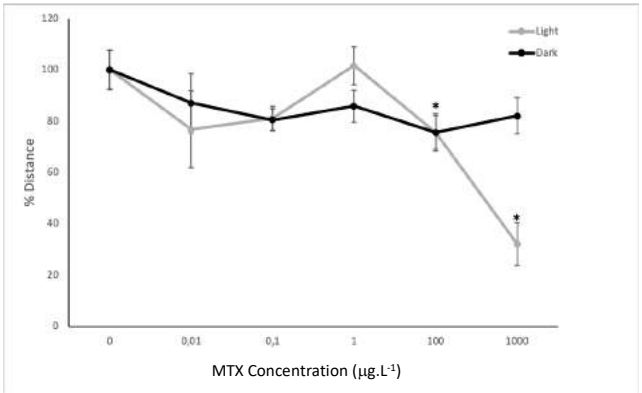


B. Medium movements

1



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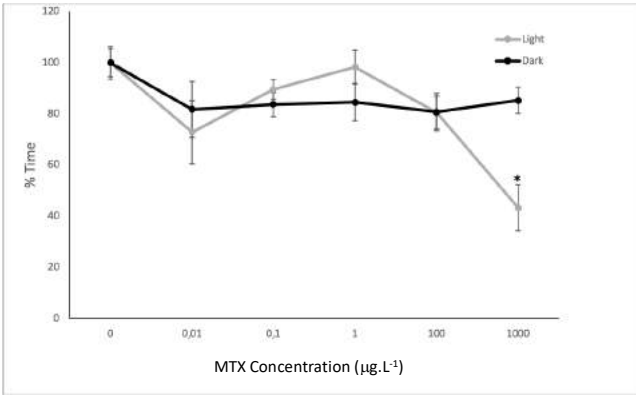
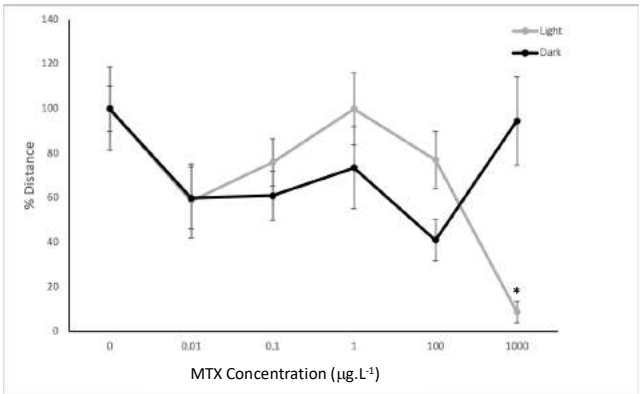


C. Rapid movements

1



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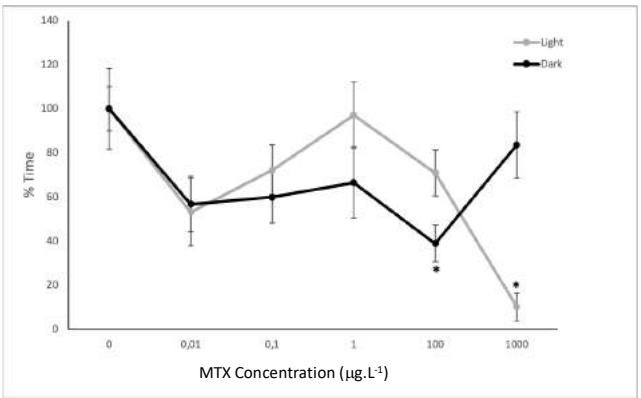
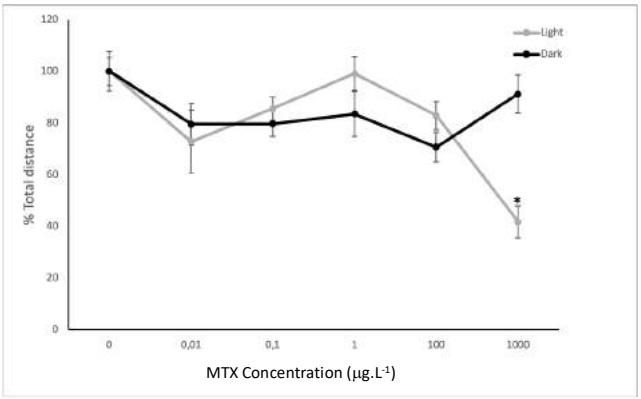


D. Total distance and total time

1



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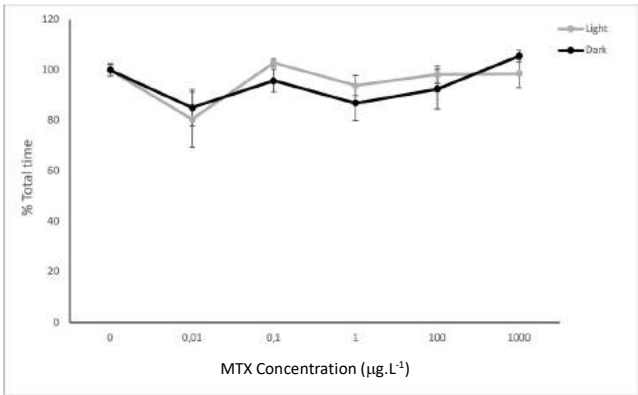
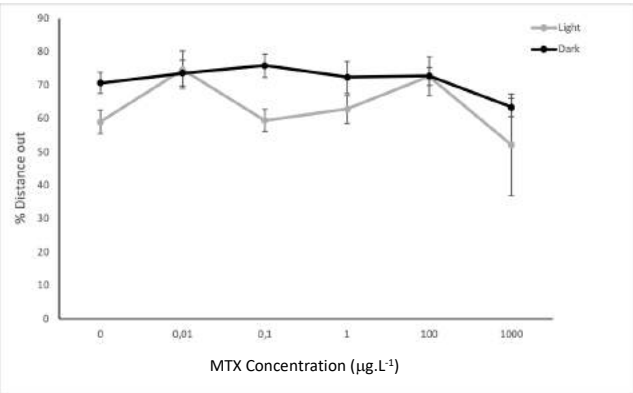


E. Distance out and time out

1



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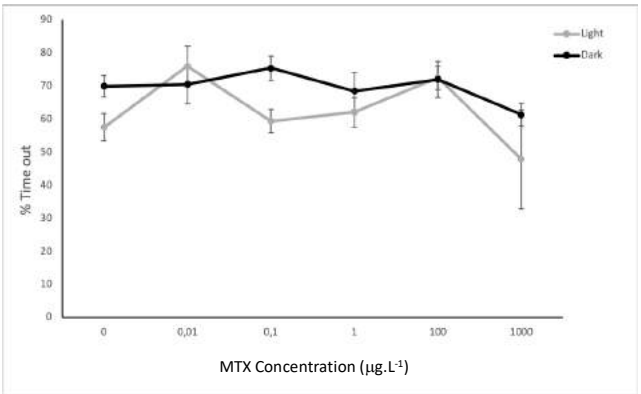


Figure 11. Evaluation of locomotor activity and thigmotaxis of zebrafish after second exposure to methotrexate. **A-** Inactivity/slow movements (A1- Distance; A2- Time); **B-** Medium movements (B1- Distance; B2- Time); **C-** Rapid movements (C1-Distance; C2- Time); **D-** Total distance and total time (D1- Total distance; D2- Total time); **E-** Distance out and time out (E1- Distance out; E2- Time out). (*$p<0.05$)

3.4. Biochemical endpoints

In the first test with MTX, no significant differences were observed in any of the biomarkers.

In the second test with MTX, differences were observed only in two biomarkers, CAT and GST. In CAT, significant differences were observed only in the concentration of 1 μg.L^{-1}, where the CAT activity is much higher than the control (**Figure 12, A**). In terms of GST, activity is lower than control at concentrations 0.01, 10 and 100 μg.L^{-1}, but significant differences were only observed for 100 μg.L^{-1} (**Figure 12, B**).

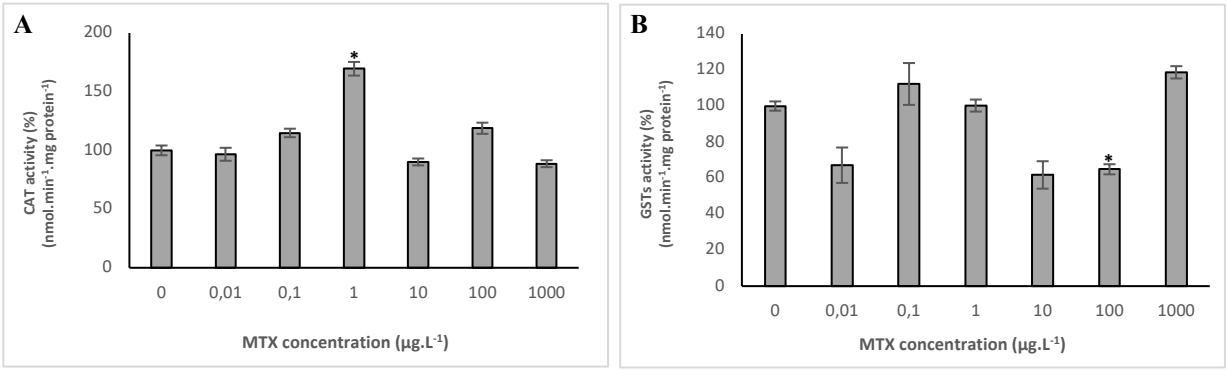


Figure 12. Evaluation of biomarkers after second zebrafish exposure to methotrexate. **A-** CAT activity; **B-** GST activity. (*p<0.05)

4. Results of 5-Fluorouracil analysis

4.1. Heartbeat rate

The analysis of the cardiac activity (beats/min) at 48 hpf revealed significant differences in terms of heartbeat at concentrations of 1, 10 and 100 μg.L^{-1} (**Figure 13**), where the heartbeat rate was lower than control. The number of beats per minute in all tested conditions varied between 40 and 120 (p<0.05).

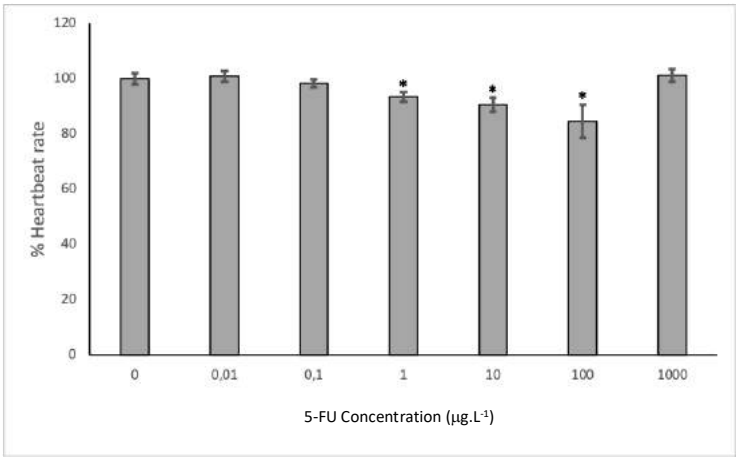


Figure 13. Heart beats measured at 48 hpf in zebrafish larvae after exposure to 5-fluorouracil. Values are expressed in beats per minute. (*$p<0.05$)

4.2. Fish Embryo Toxicity (FET) assay

At 48 hours exposure, the concentrations 0.01 µg.L^{-1} elicited higher mortality than control (**Figure 14, A**). After 96 hours exposure, the larvae equilibrium was evaluated, with a lower equilibrium in all concentrations compared to the control, but only the concentration of 1 µg.L^{-1} showed significant differences once the larvae was lower equilibrium than in the other concentrations (**Figure 14, B**). The zebrafish edema is the only parameter that shows significant differences in the three consecutive days (after 48, 72 and 96 hours of exposure). After 48 and 72 hours of testing, edema in the zebrafish was observed at 10 and 100 µg.L^{-1} (**Figure 14, C**; **Figure 14, D**), and after 96 hours of testing also the concentration of 1 µg.L^{-1} showed edema in larvae (**Figure 14, E**). However, significant differences were observed only in the concentration of 10 µg.L^{-1}.

Although no significant differences were observed in other parameters, such as malformation and tail deformation, **Figure 15** shown some pictures of zebrafish taken during the test, with these alterations. Pictures with alterations where significant differences were observed, are also represented (**Figure 15, E and Figure 15, F**), such as zebrafish edema.

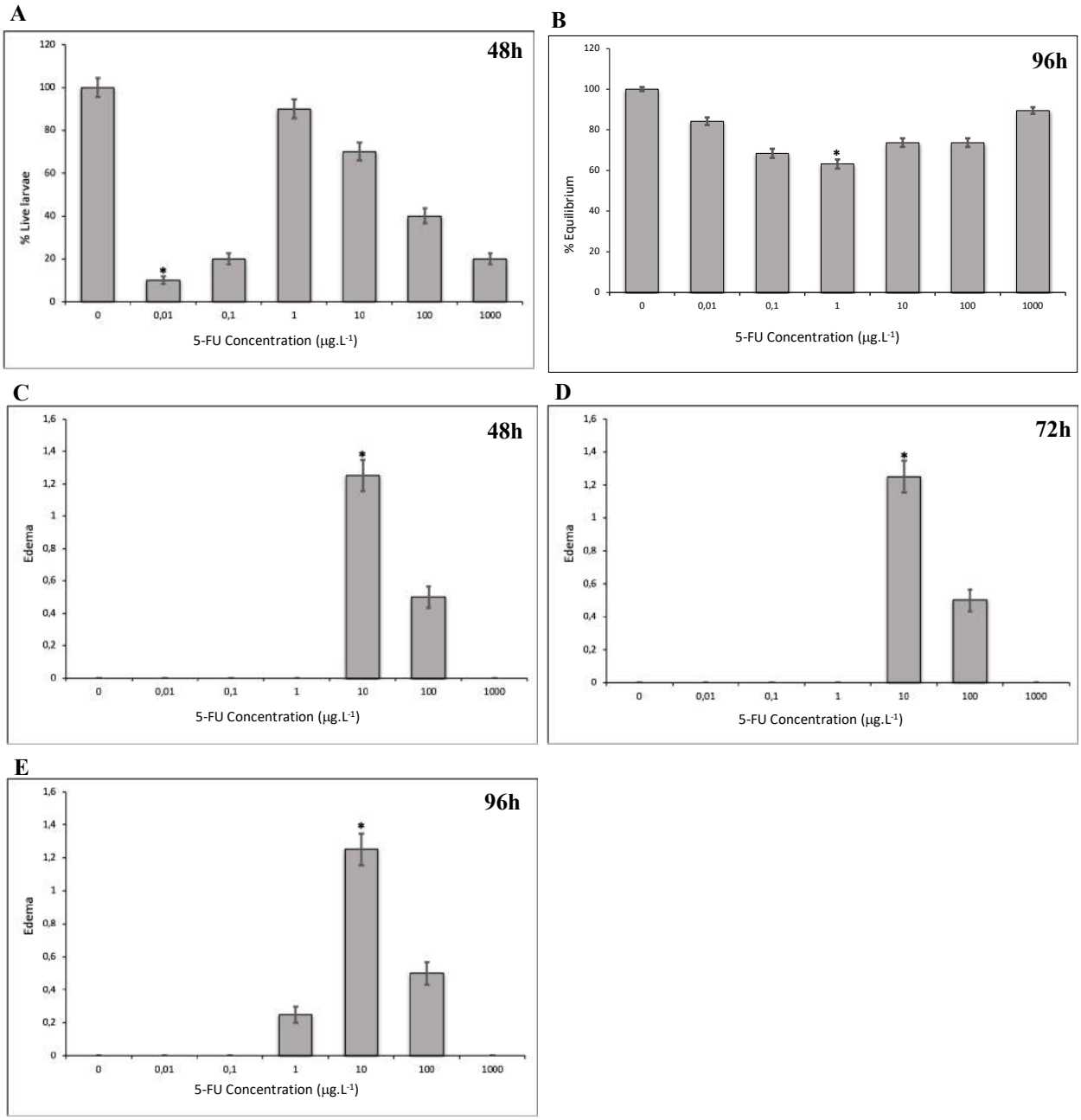


Figure 14. Evaluation of different parameters in zebrafish embryos up to 96 hours of exposure to 5-fluorouracil. **A-** Live larvae; **B-** Zebrafish's equilibrium; **C, D, E-** Edema. (*p<0.05)

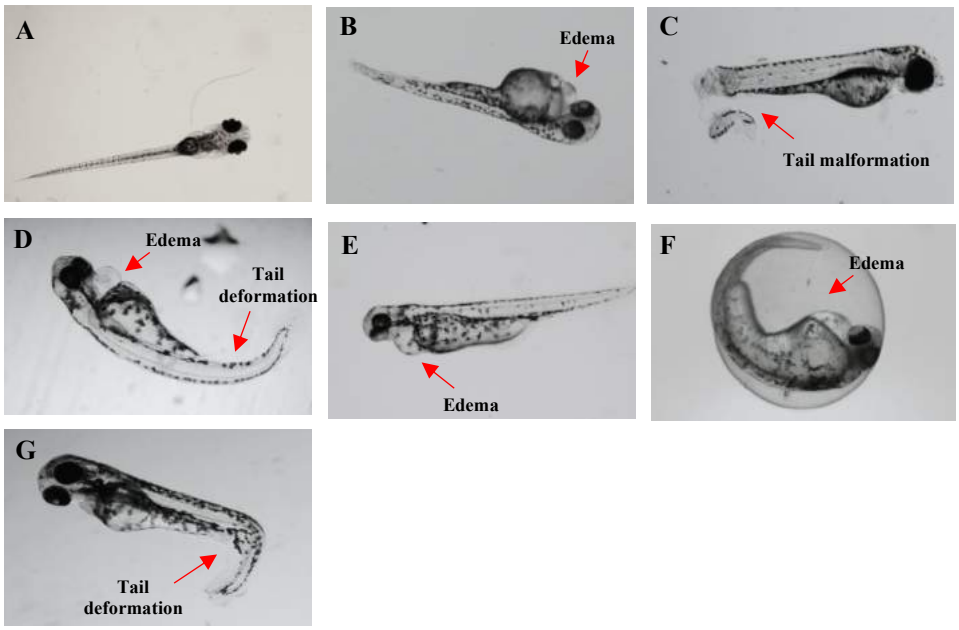


Figure 15. Representative specimens of zebrafish pictures after exposure to 5-fluorouracil, with 100x ampliation, taken during the FET test. **A-** Control; **B-** Edema ([5-FU]= 0.01 µg.L^{-1}); **C-** Tail malformation ([5-FU]= 0.1 µg.L^{-1}); **D-** Edema and Tail deformation ([5-FU]= 1 µg.L^{-1}); **E-** Edema ([5-FU]= 10 µg.L^{-1}); **F-** Edema ([5-FU]= 100 µg.L^{-1}); **G–** Tail deformation ([5-FU]= 1000 µg.L^{-1}).

4.3. Behavior assay

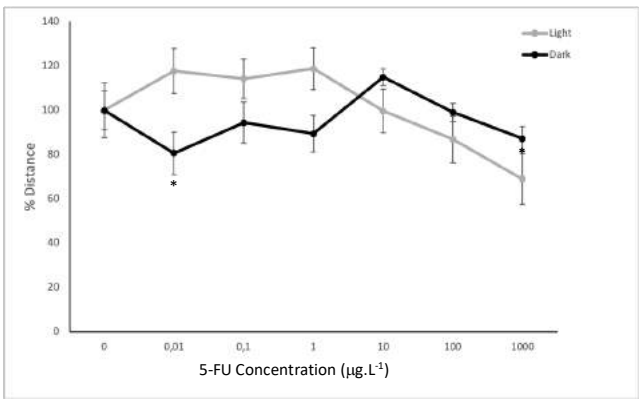
Fish exposed to 5-fluorouracil in the embryonic stage were subjected to 1 cycle of light and dark periods to assess locomotor activity, thigmotaxis and habituation. When analyzing the results, the reaction to the sudden changes in light conditions appeared to be depend on the concentration of 5-fluorouracil to which fish were pre-exposed.

In this test, significant differences were observed in inactivity/slow, medium and rapid movements. In inactivity/slow movements (**Figure 16, A**), significant differences were observed only in terms of distance (**Figure 16, A1**), where an initial decrease in distance was noticeable compared to the control in the dark phase, with significant differences in the concentration 0.01 µg.L^{-1}, with a more pronounced decrease. In the light phase, the distance swam was not significantly affected in all concentrations except for the highest concentration

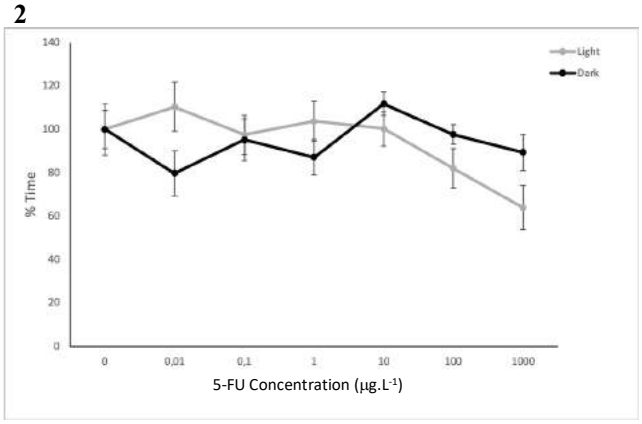
tested (1000 μg.L^{-1}), where a marked decrease was observed compared to the control. Over time, there were no significant differences. In terms of medium movements (**Figure 16, B**), there were significant differences in both distance and time at the same concentrations, observed only in the light phase in the three lowest concentrations (0.01, 0.1 and 1 μg.L^{-1}), where a marked increase in relation to the control and the dark phase condition was observed. Considering rapid movements (**Figure 16, C**), the pattern of response was similar to the medium movements. Regarding total distance (**Figure 16, D1**), the same occurs in medium (B) and rapid movements (C), with significant differences only detected in the three lowest concentrations (0.01, 0.1 and 1 μg.L^{-1}) tested, in light phase, where the values were greater than the control. However, in terms of total time (**Figure 16, D2**), significant differences were observed only in 0.01 μg.L^{-1} in the light phase. In terms of distance out and time out (**Figure 16, E**), significant differences were observed only in distance swam in the outer area, in the lowest concentration tested (0.01 μg.L^{-1}) in the dark phase, with values higher than in the control (**Figure 16, E1**).

A. Inactivity/slow movements

1

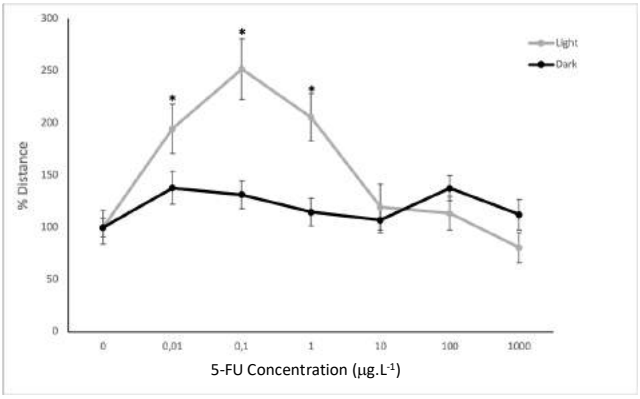


2



B. Medium movements

1



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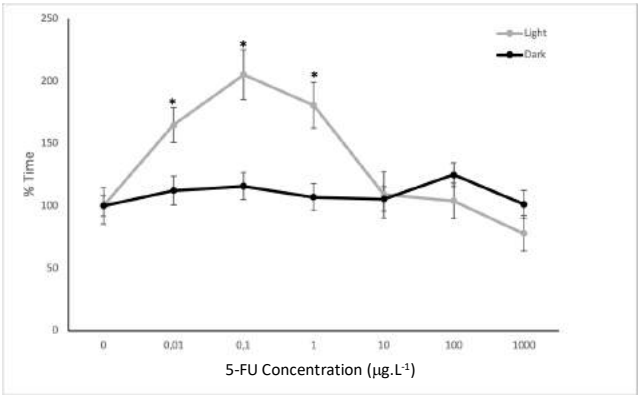
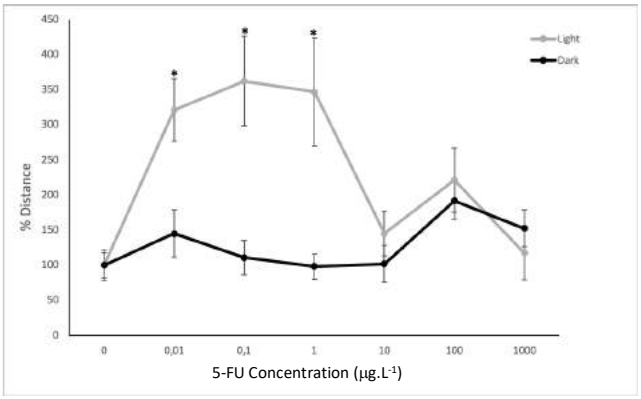


C. Rapid movements

1



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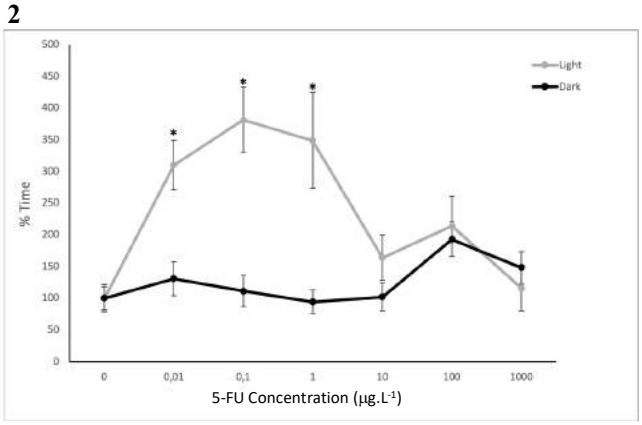
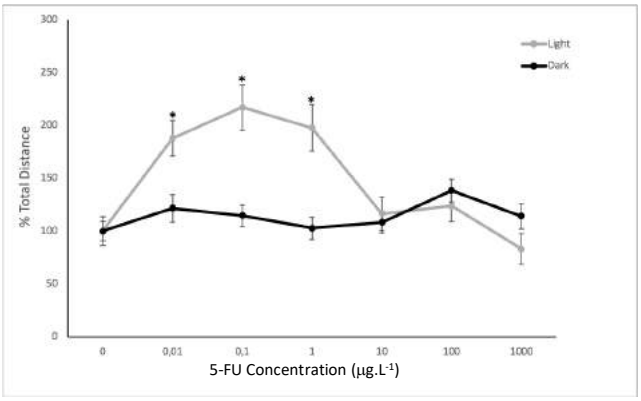


D. Total distance and total time

1



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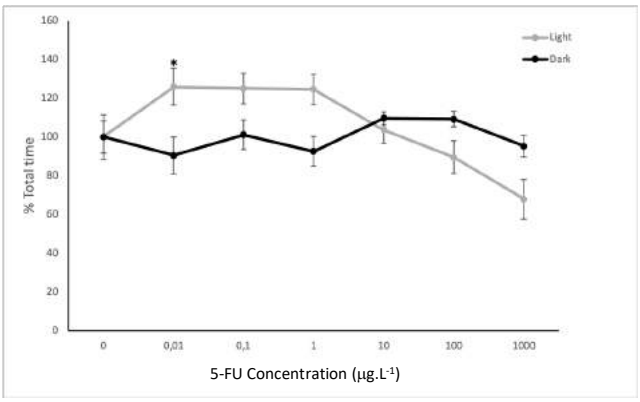
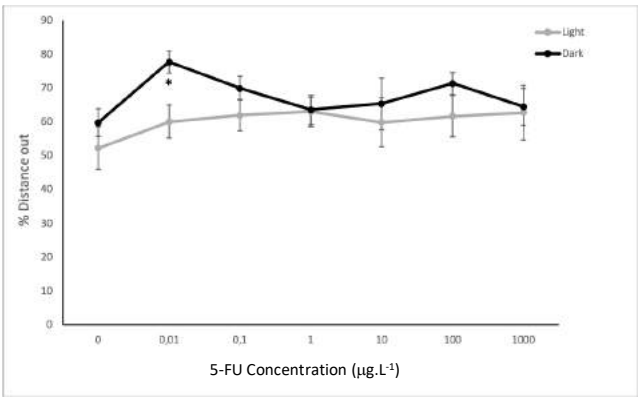


E. Distance out and time out

1



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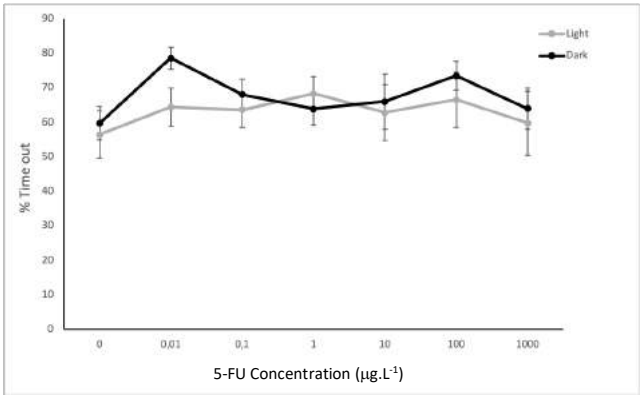


Figure 16. Evaluation of locomotor activity and thigmotaxis of zebrafish after exposure to 5-fluorouracil. **A-** Inactivity/slow movements (A1- Distance; A2- Time); **B-** Medium movements (B1- Distance; B2- Time); **C-** Rapid movements (C1-Distance; C2- Time); **D-** Total distance and total time (D1- Total distance; D2- Total time); **E-** Distance out and time out (E1- Distance out; E2- Time out). (*p<0.05)

4.4. Biochemical endpoints

In the test with 5-FU, differences were observed only in terms of CAT activity. In this biomarker, significant differences were observed only in the concentration of 10 µg.L^{-1}, where the CAT activity is higher than the control (**Figure 17**).

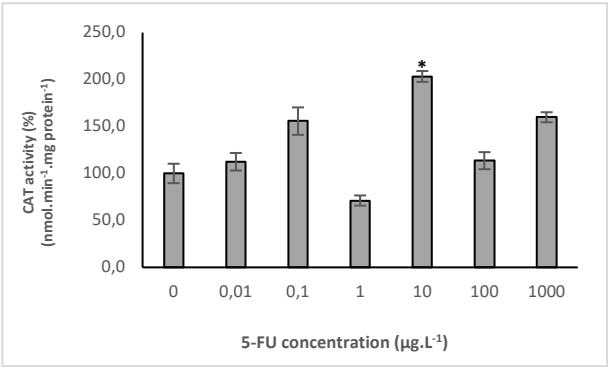


Figure 17. Evaluation of CAT activity after zebrafish exposure to 5-fluorouracil. (*p<0.05)

5. Results of Mixture (Methotrexate and 5-Fluorouracil) analysis

5.1. Heartbeat rate

The analysis of the cardiac activity (beats/min) at 48 hpf revealed no significant differences between groups in terms of heartbeat (**Figure 18**). The number of beats per minute in all tested conditions ranged between 92 and 116 ($p<0.05$).

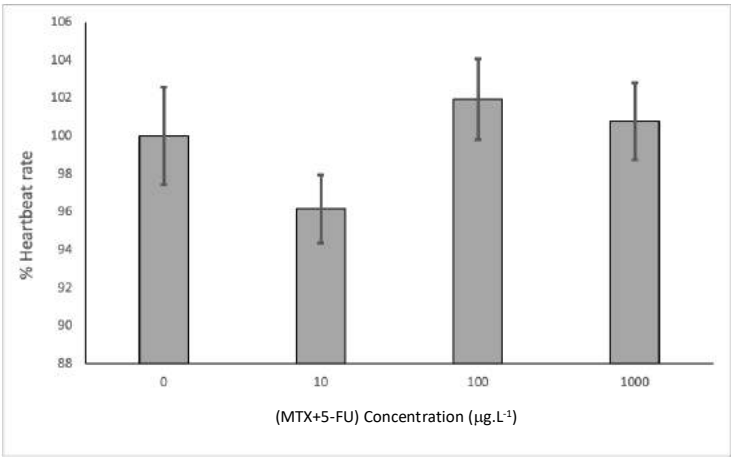


Figure 18. Heart beats measured at 48 hpf in zebrafish larvae after exposure to the combination of methotrexate and 5-fluorouracil. Values are expressed in beats per minute. (*$p<0.05$)

5.2. Fish Embryo Toxicity (FET) assay

In the test of the mixture of methotrexate with 5-fluorouracil, the parameters evaluated with significant differences when compared to the control were tail deformation, zebrafish's malformation and zebrafish's edema. After 24 hours exposure, deformations in the tail of some embryos in all concentrations tested were noted, but significant differences were observed only in organisms exposed to 100 $\mu g.L^{-1}$, which presented more deformations than the control (**Figure 19, A**). After 48 hours, significant differences were observed in terms of malformations (**Figure 19, B**) and presence of edema (**Figure 19, C**) of embryos exposed to 10 $\mu g.L^{-1}$.

Figure 20 presents some pictures of the zebrafish taken during the test, where some alterations mentioned above are visible.

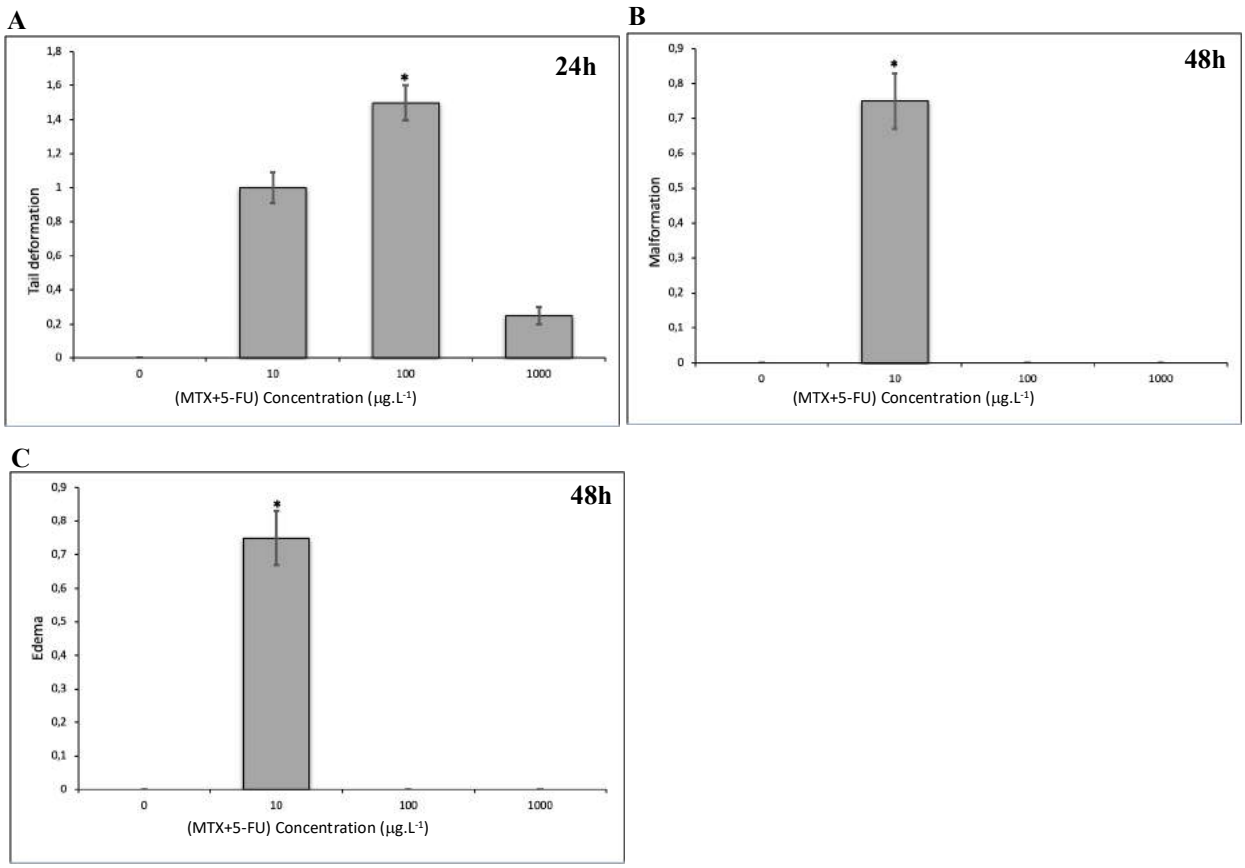


Figure 19. Evaluation of different parameters in zebrafish embryos up to 96 hours of exposure to combination of methotrexate and 5-fluorouracil. **A-** Tail deformation; **B-** Zebrafish's malformation; **C-** Edema. (*p<0.05)

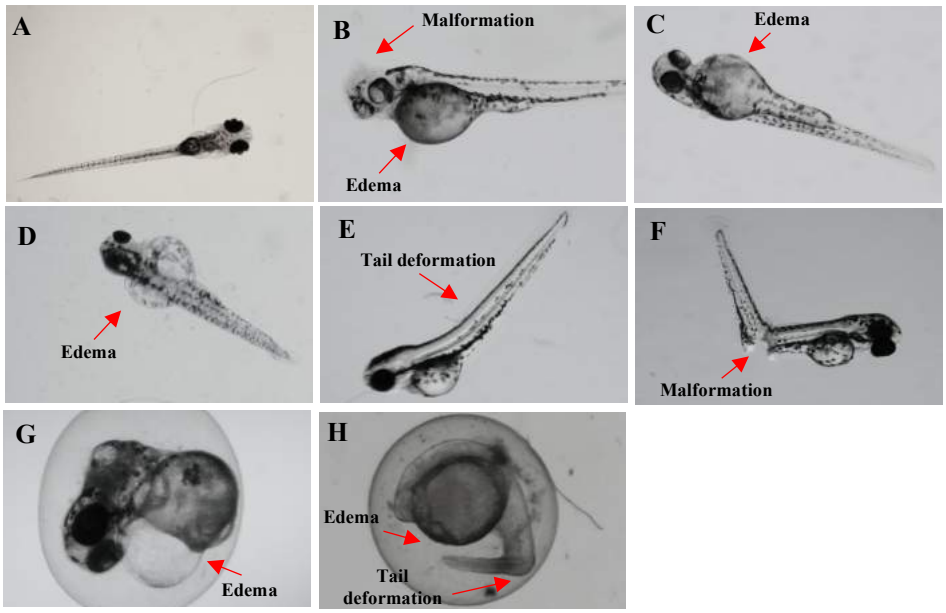


Figure 20. Representative specimens of zebrafish pictures after exposed to the combination of methotrexate and 5-fluorouracil, with 100x ampliation, taken during the FET test. **A-** Control; **B-** Edema and Malformation ([MIX]= 10 µg.L^{-1}); **C-** Edema ([MIX]= 10 µg.L^{-1}); **D-** Edema ([MIX]= 10 µg.L^{-1}); **E-** Tail deformation ([MIX]= 10 µg.L^{-1}); **F-** Tail malformation ([MIX]= 10 µg.L^{-1}); **G**– Edema ([MIX]= 100 µg.L^{-1}); **H-** Edema and Tail deformation ([MIX]= 1000 µg.L^{-1}).

5.3. Behavior assay

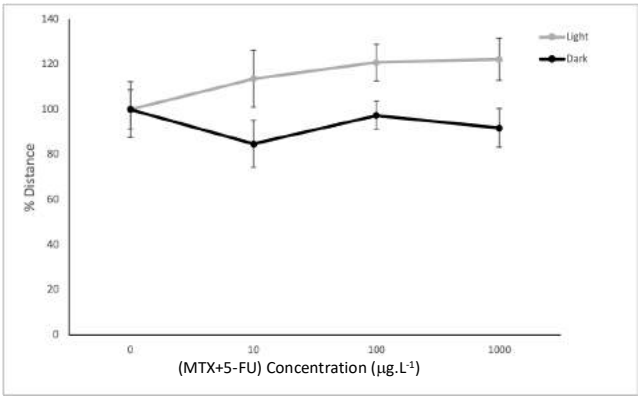
The reaction to sudden changes in light conditions was affected significantly by 100 and 1000 µg.L^{-1}.

In inactivity/slow movements (**Figure 21, A**), no significant differences were observed, neither on distance nor time. In medium movements (**Figure 21, B**), significant differences were observed in the light phase in the two highest concentrations (100 and 1000 µg.L^{-1}), both in distance and time, with increased values compared to control. In terms of rapid movements (**Figure 21, C**), the response pattern was identical to medium movements. Significant differences were observed in the light phase in organisms exposed to 100 µg.L^{-1}, which presented higher values than the control. Considering total distance (**Figure 21,**

D1), in the light period, significantly higher values than control were observed in organisms exposed to 100 and 1000 µg.L^{-1}. However, in terms of total time no significant differences were observed (**Figure 21, D2**). In distance out (**Figure 21, E1**), significant differences in the dark phase were observed for all concentrations (10, 100 and 1000 µg.L^{-1}), with values being higher than control. In terms of time out there were no significant differences (**Figure 21, E2**).

A. Inactivity/slow movements

1



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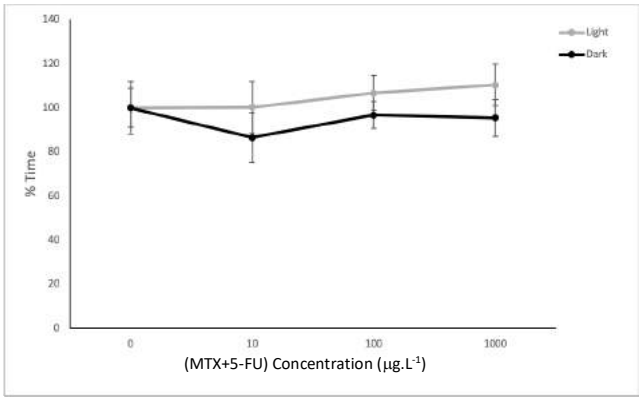
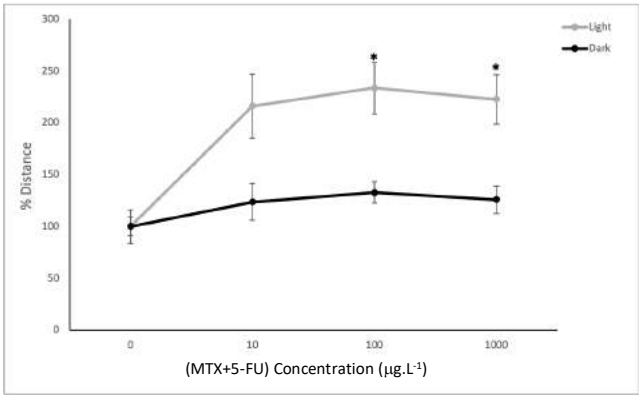


B. Medium movements

1



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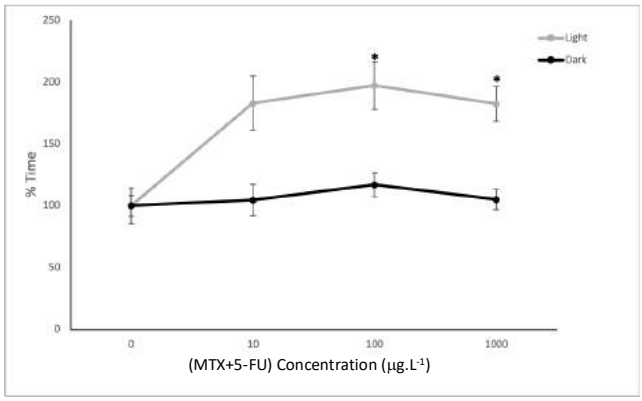
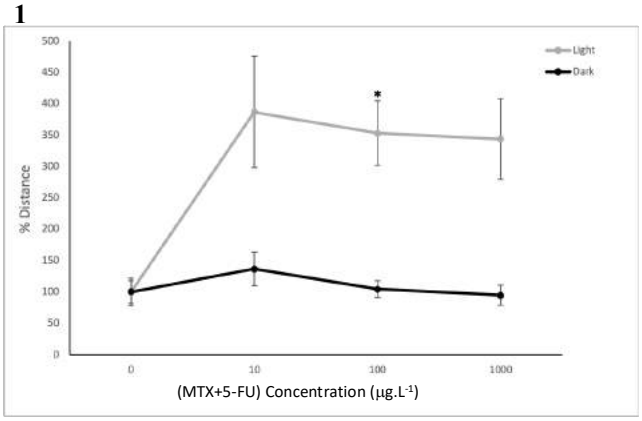


C. Rapid movements

1



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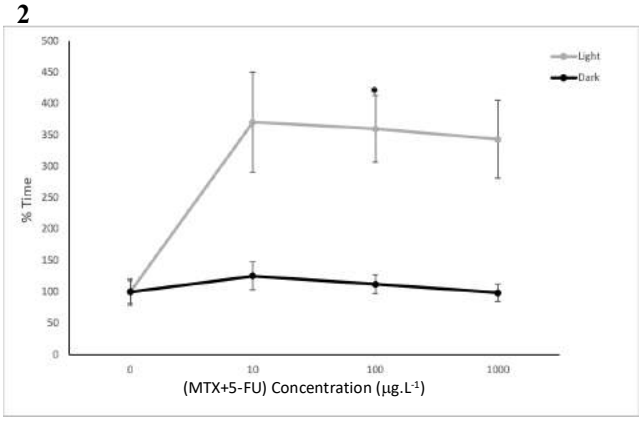
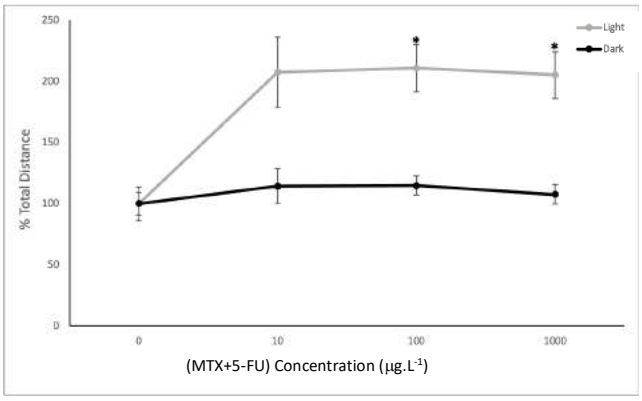


D. Total distance and total time

1



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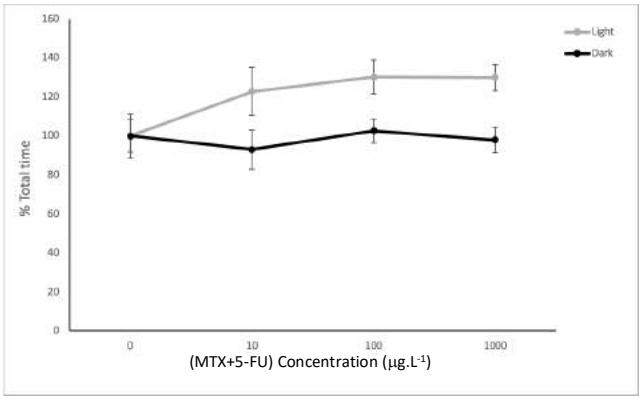
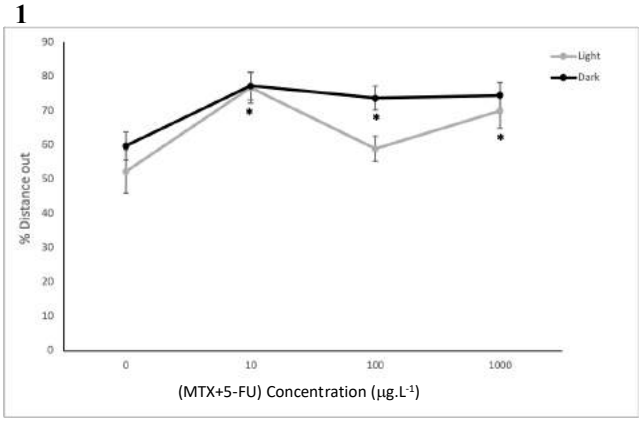


E. Distance out and time out

1



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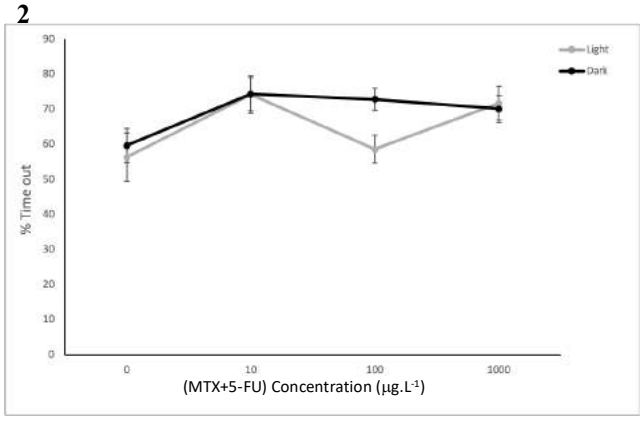

Figure 21. Evaluation of locomotor activity and thigmotaxis of zebrafish after exposure to the combination of methotrexate and 5-fluorouracil. **A-** Inactivity/slow movements (A1- Distance; A2- Time); **B-** Medium movements (B1- Distance; B2- Time); **C-** Rapid movements (C1-Distance; C2- Time); **D-** Total distance and total time (D1- Total distance; D2- Total time); **E-** Distance out and time out (E1- Distance out; E2- Time out). (*$p<0.05$)

5.4. Biochemical endpoints

In the test with the mixture of methotrexate and 5-fluorouracil, no significant differences were observed in any of the tested biochemical biomarkers.

6. Summary of results

In this work it was performed four different essays, evaluating parameters regarding development, behavior and biochemical biomarkers. In **Table 1** is shown all the parameters of each essay with significant differences to the control ($p<0.05$), with the respective concentrations for the significant differences, represented in $\mu g.L^{-1}$.

Table 1. Summary of the results of the parameters evaluated in the four tests performed, methotrexate performed in light, methotrexate performed in dark, 5-fluorouracil and combination of methotrexate and 5-fluorouracil.

		MTX (Light) ($\mu g.L^{-1}$)	**MTX (Dark)** ($\mu g.L^{-1}$)	**5-FU** ($\mu g.L^{-1}$)	**Mixture** ($\mu g.L^{-1}$)
Heartbeat rate (48h)			↓ 0.01 ↑ 1000	↓ 1, 10 and 100	
FET (96h)	Tail Deformation		↑ 1 and 10		↑ 100
	Deformation		↑ 100		
	Malformation				↑ 10
	Transparent Larvae	↑ 10, 100 and 1000	↑ 1, 100 and 1000		
	Pigmentation	↑ 0.01, 0.1 and 10			
	Edema			↑ 10	↑ 10
	Light Eyes		↑ 1000		
	Spontaneous movement	↑ 10	↓ 1 and 10		
	Live Larvae	↓ 10 and 100		↓ 0.01	
	Equilibrium			↓ 1	
Behavior (120h)		↓ 1000 (Light) Thigmotaxic response: 1000 (Ligth)	↓ 1000 (Light) No thigmotaxic response	↑ 0.01, 0.1 and 1 Thigmotaxic response: 0.01 (Dark)	↑ 100 and 1000 Thigmotaxic response: 10, 100 and 1000 (Dark)
Biomarkers (96h)	CAT		⊕ 1	⊕ 10	
	AChE				
	GST		⊖ 100		
	LDH				

Subtitle:

- No significant differences
- ↑ Increased values
- ↓ Decreased values
- ⊕ Induction
- ⊖ Inhibition

7. Discussion

The presence of pharmaceuticals residues in the aquatic environment has been increasingly worrisome due to the potential effects they can cause in non-target organisms after prolonged exposures, as demonstrated by several laboratory studies, even in low concentrations, through different modes of action (15). In this work, the model organisms used were zebrafish embryos, which may absorb small molecules diluted in surrounding water through their gills, skin and gut. The absorption of the compounds depends on the physical-chemical properties of each compound and the results obtained in aqueous exposure experiments, may not always be true, but false-negatives, if there is low absorption of the compound (16).

Different endpoints were assessed to test the toxicity of these compounds, MTX and 5-FU, and the possible effects that they could provoke in non-target organisms. FET in general reveled that, both compounds individually and in combined exposures, can caused development deformations like deformed tail as well as edema mainly in the heart. The deformations and malformations observed in zebrafish, such as the curvature of the body mainly in the tail, has been previously reported in assays with 5-FU. For MTX, effects on body curvature have not been tested (17). Both deformations and malformations can be related to the teratogenic effects that can be caused by both drugs, since it is one of the side effects and consists of the malformations of the embryos. In the present study, embryos with light eyes were observed after second exposure to MTX. Deformations in the eyes had already been reported in zebrafish embryos exposed to concentrations of 5-FU higher than 1000 $mg.L^{-1}$ (17). Deformations in the eyes are known as uveal coloboma and have also been reported in humans as a hereditary malformation (17). This study highlighted that there are similarities between zebrafish and human (18). Other studies showed changes in the liver, kidneys and heart at 300 $mg.L^{-1}$ of 5-FU, as seen in this study with the edema in the heart, which may be related to the cardiac toxicity that can be caused by these drugs. A study carried out with adult larvae observed changes in the liver and kidneys, such as lipidosis, liver atrophy and degeneration of the kidney tubular epithelium (tubulonephrosis) when they were exposed to concentrations above 1 $\mu g.L^{-1}$ of 5-FU (19). Regarding MTX, differences at the hepatic level (mild to moderate hepatic changes with congestion and hydropathic hepatocellular degeneration) and cardiac (mild myocardial fiber degeneration) have been

reported in Wistar rats, exposed, for 45 days, to this drug (20). Both in our experiment, as in the studies referred to previously, problems were detected at the heart level, in different organisms (zebrafish and Wistar rats), which may be an indication of MTX toxicity. The toxicity caused by MTX may be related to the mechanism of action of this drug since there is a depletion of tetrahydrofolate (THF) and its derivatives, interfering with the synthesis of DNA and in doing so, affecting the cell division and proliferation cycle which can be associated with reported cytotoxicity and potential teratogenicity (21). MTX is a photosensitive drug and the different changes observed between the first and the second test with MTX are possibly related to the exposure of the first test to light (22). This factor may justify the presence of transparent and pigmented larvae in the first test in the presence of light, not being observed in the second test, since exposure to light after the administration of MTX can cause changes in the skin pigmented in relation to humans (23). In addition, significant differences were observed in the deformed larvae only in the second test in the absence of light, which may indicate that MTX has been degraded in the first test over time with the presence of light, not causing so many effects. Mixing drugs in the environment is increasingly of greater concern, as these mixtures can cause more serious effects on non-target organisms. An experiment carried out by *Róbert Kovács, 2016* with a mixture of different cytostatic compounds (CP, 5-FU, IF and CDDP), showed more serious changes than when these compounds act individually, both in zebrafish and in human cells (13). The same was verified in the present study, since the changes in the embryos and in the larvae were more severe and noticeable and occur in all concentrations, unlike the embryos and larvae exposed to the compounds individually at the same concentrations as were present in the mixture. In combinatory treatments, drug interaction can be divided into three classifications: antagonistic, additive and synergistic (24). The antagonistic interaction occurs when the effectiveness is reduced since one of the agents used counteracts the action of the other agent. The additive approach results in a less significant reduction of applied dosages when used in combination or alone. Finally, synergism occurs when the combined action of both agents is more effective (24). Therefore, the mixture of MTX with 5-FU is considered a synergistic interaction between these compounds, since the combined action was more effective than the action of the individual compounds causing more effects. These results contribute to the evidence that the maximum concentration levels of compounds allowed in the environment, established based on toxicological data for individual

compounds, may not be low enough to protect the environment against the combined effects of mixtures of pollutants (13).

Behavioral parameters have been considering a sensitive tool to assess the effects of xenobiotics. Changes in behavior during swimming can have ecological consequences, leading to changes in the prey-predator relationship and consequently reducing competitive advantage (25). Alternating periods of light and dark have been used in zebrafish larvae as a startle to evaluate the stress response, with larvae increasing activity (26). In this work, four behavioral tests were performed. Two with MTX, one with 5-FU and finally another with the mixture of the two drugs. In the both behavioral test with MTX, a lower swimming distance was observed in the light in relation to the control, mainly in the highest concentration (1000 $\mu g.L^{-1}$), thus showing hypoactivity (reduced swimming distance) of fish when exposed to light. This type of behavior is similar to another experience, where after the exposure of the zebrafish embryos to MTX, a decrease in the locomotor activity of the fish was observed (27). For the 5-FU, the distance swam by the tested larvae was higher in light at the lowest concentrations (0.01, 0.1 and 1 $\mu g.L^{-1}$), showing hyperactivity of the larvae, which may be indicative of stress after light stimulation. After concentration 10 $\mu g.L^{-1}$, the situation is reversed and the distance swam by the larvae is lower when they are exposed to light, thus conferring hypoactivity of the fish as with MTX. In the mixture, the distance swam by the larvae is always greater in the light than in the dark, which can indicate anxiety (freezing, hyperactivity and erratic swimming). This may be related to the mixing of drugs, since mixtures usually have more serious effects than compounds when administered individually. Thigmotaxis is measured by the percentage of movement in the outer zone of the tank and refers to the tendency of an animal to remain close to the walls, avoiding the center of the aquarium, when it is placed in a new environment. In the case of fish, thigmotaxis is considered a measure of anxiety (28). When analyzing the distance in the external zone, an increase in the distance was observed comparing to the control when the fish was exposed to MTX, 5-FU and the mixture, indicating that there was a thigmotaxic response, which is related to the anxiety of the fish when they were exposed to the drug. The effects of exposure to MTX and 5-FU were evident in the behavior of fish, since they showed hypoactivity when exposed to MTX and to the highest concentrations (10, 100 and 1000 $\mu g.L^{-1}$) of 5-FU, and hyperactivity when exposed to the lowest concentrations (0.01, 0.1 and 1 $\mu g.L^{-1}$) of 5-FU and to mixture, indicating anxiety.

Accurate quantification of heartbeats in fish models, such as zebrafish, is an important readout to study pharmacology, cardiovascular biology and disease states (29). Generally, in this work, the heartbeat rate of the fish was superior to the control, which can be related to the anxiety observed in the behavior, since one of the effects of anxiety is the increase in heartbeat. The increase in beats was not observed at concentrations 1, 10 and 100 $\mu g.L^{-1}$ of 5-FU and at the lowest concentration (10 $\mu g.L^{-1}$) of the mixture, which may be related to the fact that edema in the heart was observed in embryos at these concentrations, since in these cases the heartbeat rate tends to be very low.

The ROS detoxification mechanisms are associated with a combination of multiple enzymes such as CAT, which can be considered as the first line of defense against the deleterious effect provoked by excessive production of ROS in response to chemical exposure (30). Regarding CAT activity our results shown a significant induction in organisms exposed to 1 $\mu g.L^{-1}$ MTX and the concentration of 10 $\mu g.L^{-1}$ of 5-FU. This induction can be explained by the catalytic activity of CAT enzyme, to convert hydrogen peroxide (H_2O_2) into oxygen (O_2) and water H_2O (31).

The activity of AChE is often used as a biomarker of environmental pollution, since it is inhibited by neurotoxic compounds (15). In this work, there were no differences in AChE activity in any of the tests, which can be explained by the fact that they are short tests and with relatively low concentrations compared to the concentrations administered in a human with a tumor, since MTX is neurotoxic when it is administered for 2 to 14 days in a row at low doses (5-50 $mg.m^{-2}$) or at high doses (> $1g.m^{-2}$). For 5-fluorouracil, neurotoxicity is rare (32,33).

Glutathione S-transferase (GST) are among the phase II biotransformations enzymes and primary antioxidant enzymes and are first-line indicators of the antioxidant state (34). In this study GST activity was inhibited at concentration of 100 $\mu g.L^{-1}$ of MTX. This inhibition could be due to an impairment of the GST pathways in response to a substantial stress increase or decreased levels of reduced glutathione.

In relation to LDH, an exercise situation requiring more energy and/or stress is reflected by an increase in LDH activity (35). In this study, there were no changes in LDH, which may justify the behavior results, since in general the distance swam by the fish was less than or approximately equal to the control, not justifying energy expenditure. No articles with previous tests were found that studied biomarkers after exposure to MTX and 5-FU.

These results demonstrated that 5-FU appear to be more toxic than MTX, since it caused more side effects. Both drugs, individually and in combination, mainly affect the heart and tail of zebrafish, causing edema in the heart and deforming the tail.

8. Final considerations

This study was performed in order to gain insight on the potential effects of cytostatic drugs in non-target organisms.

The results obtained allowed an understanding of the mechanisms of action of 5-fluorouracil and methotrexate, and allowed us to understand the impact of these cytotoxic substances in zebrafish.

In summary, the main results allowed to conclude that exposure to methotrexate and 5-fluorouracil has harmful effects mainly on the heart and tail and can cause anxiety in fish. 5-Fluorouracil was shown to be more toxic than methotrexate since it had effects in practically all concentrations, whereas in exposure with methotrexate the changes occurred mostly at the highest concentration. This situation can be worrying, due to the high use and high prescription of 5-fluorouracil. The mixture of these two drugs, caused more severe effects on fish, which shows that the mixture of substances can be more dangerous. The results obtained during this work may represent the potential effects on human health and the potential environmental effects leading to an alert on the use of these substances and their release into the environment.

9. Future Perspectives

This study is expected to contribute to the current scientific knowledge on the effects of cytostatic drugs to non-target organisms. The obtained data may also contribute to recommendation to use other alternatives in cancer treatments and/or improvements of wastewater treatment plants. The data obtained in this study will also be used as basis to future long-term studies.

It would be important in the future to carry out further studies with cytostatic drugs assessing other endpoints, like DNA damage, the evaluation of subsequent generations, chronic exposures and responses to molecular levels, since the incidence of cancer in the population is increasingly high, which leads to an increase in the consumption of cytostatic drugs and, consequently, a greater release of these drugs into the environment.

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