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Developmental toxicity and epigenetic changes induced by pharmaceuticals in zebrafish (*Danio rerio*)

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INTRODUCTION

The zebrafish model

Native to the freshwater pools and streams in Southern Asia, *Danio rerio* is a small tropical teleost fish belonging to the family *Cyprinidae*, under the class *Actinopterygii* (Meyer et al., 1993; Arunachalam et al., 2013). It gets the common name "zebrafish" from the longitudinal dark blue and silver-yellow stripes on either side of its compressed body, extending to the anal fin and onto the caudal fin rays of the tail (Grunwald and Eisen, 2002; Schilling, 2002; Engeszer et al., 2007).

Adult female and male zebrafish are easily recognizable from their secondary sexual characteristics, such as body shape and pigmentation. Females show a rounder belly full of eggs, a visible oviduct near the anal fin and they tend to have blue and white colouring. In contrast, males are sleeker and have a bright yellow pigmentation, due to the testosterone (Yu et al., 2018).

Zebrafish are easy to breed and relatively inexpensive to keep, while embryos are transparent and have rapid development. Moreover, zebrafish share many biological features, genes, developmental processes, anatomy and physiology with their human counterparts (Zhang et al., 2003). Given these strengths, the zebrafish has recently become a popular model organism for scientific research in several fields, including Biomedicine, Toxicology, Environmental Science, Biotechnology and Aquaculture (Lieschke & Currie, 2007).

The pioneer works that defined zebrafish as a versatile model organism date back to the late 1960s, when George Streisinger first realized the potential of this small vertebrate and chose it for his genetic studies at University of Oregon. The publishing by Streisinger and his colleagues in the early 1980s, along with the evident advantages offered by zebrafish, contributed to its widespread adoption as a model organism for developmental biology and other fields (Lieschke & Currie, 2007; Ablain & Zone, 2013; Giannaccini et al., 2014).

The identification of thousands of zebrafish mutant lines in the early 1990s (Mullins & Nüsslein-Volhard, 1993; Driever et al., 1994) and the sequencing of the zebrafish genome, performed from 2001 to 2013 (Howe et al., 2013) have provided a clearer understanding of critical genomic features. Thus, the zebrafish research community has grown exponentially over the last decades, leading to a dramatic rise in publications about this animal model.

Zebrafish development and life cycle

Being a poikilothermic organism, incubation temperature affects developmental rate of zebrafish. In 1995, Kimmel and his collaborators firstly described the zebrafish developmental stages based on morphology and timing as standard hours post fertilization (hpf) at 28.5°C (Kimmel et al., 1995). Today, all papers discussing on zebrafish developmental stages refer to this work.

After fertilization, cytoplasm flows towards the animal pole to separate the blastodisc from yolk. The first meroblastic division starts the cleavage period at about 45 minutes post-fertilization. After this point, cell cycles take around 15 minutes and successive cell divisions occur synchronously. At the 16-cell stage, central cells become separated from marginal blastomeres, which at first remain anchored to the yolk by cytoplasmic bridges, and subsequently collapse to form the multinucleate yolk syncytial layer (Kimmel et al., 1995; Meyers, 2018). From the 128-cell stage, the developing embryo is called blastula, and consists of a mass of cells at the animal pole. The tenth cell cycle marks the Mid-Blastula Transition, when cell divisions are longer and asynchronous compared to previous stages, and embryonic development comes under the control of the zygotic genome. The Mid-Blastula Transition also marks the start of cellular motility, and thus the beginning of morphogenetic movements leading to the onset of epiboly and the spreading of blastoderm across the yolk (Webb & Miller, 2006). From this stage onwards, the "epiboly percentage" defines the advancement of the process that leads the yolk to be surrounded completely by the blastoderm. In agreement with this nomenclature, the blastula period ends when the blastoderm margin covers the 30% of the entire distance between animal and vegetal poles, which is called the "30% epiboly stage".

Gastrulation involves epiboly continuation and simultaneous morphogenetic cell movements that generate the primary germ layers and the embryonic axis. In particular, at the 50% epiboly-stage (around 6hpf), cell involution induces a folding of blastoderm on itself. Due to this event, two germ layers, epiblast and hypoblast, can be distinguished within the so-called germ ring. At the end of gastrulation, the epiblast corresponds to the ectoderm, which will give rise to epidermis, central nervous system, neural crest and sensory placodes, while the mesoderm and endoderm will originate from the hypoblast. Subsequently, convergence of the cells at one side of the germ ring produces the so-called "shield", which marks the dorsal side of the embryo, thus allowing orientation of the dorsal-ventral axis (Kimmel et al., 1995; Webb & Miller, 2006; Meyers, 2018).

Epiboly arrests temporarily during these phenomena and it starts again from shield stage, being completed at the "Bud stage" (10hpf), when the tail bud has formed and gives the name to this stage. The tail bud appears as a swelling at the caudal end of the embryonic axis, and represents the

organizing centre for the development of the posterior trunk. The head, instead, develops from a thick region near the animal pole. At the bud stage, gastrulation is considered to be over and the embryo starts to exhibit signs of segmentation. During this phase, ectoderm, mesoderm and endoderm undergo modifications and give rise to the primary rudimentary organs. Somites appear in sequence, starting from the head region towards the tail. In the later stages of segmentation, morphogenesis produces a significant increase in length of the embryo, involving the detachment of the tail from the body and the extension of the posterior region of the yolk (Kimmel et al., 1995; Webb & Miller, 2006).

The Pharyngula period starts at 24hpf and represents the phylotypic stage, that is the stage at which the embryo shows the basic vertebrate body plan (Kimmel et al., 1995; Collazo, 2000). The term "pharyngula" refers to the pharyngeal arches that become gradually distinguishable during this phase. The embryo continues its lengthening and, consequently, the head straightens out while the head-trunk angle increases. This developmental stage also involves pigment cell differentiation and the formation of the circulatory system (Kimmel et al., 1995).

The hatching from the chorion, between 48 and 72hpf, represents the transition from the embryonic to the larval stage. Most organs of the early larva are nearly complete, except for those in the gastrointestinal tract, which develop in the time range between 72 and 96hpf (van Wijk et al., 2016). Overall, larvae undergo further body growth and specification, including swim bladder inflation, protrusion of the mouth and movements of the jaws and fins, which allow active swimming and, shortly thereafter, independent feeding from about 120hpf (Meyers, 2018).

Zebrafish take about four weeks to complete their larval period and reach about eleven millimetres of length, conventionally recognized as the marker of having reached the juvenile stage. During this time span, growth progression and terminal differentiation processes are particularly challenging to standardize, since they depend on factors such as temperature, density of population and organism-specific differences (Meyers, 2018).

Zebrafish reach sexual maturity at around three or four months of age. From this moment, zebrafish are considered adults and they usually do not exceed four centimetres in length (Reed & Jennings, 2011; Cassar et al., 2020).

It has been reported that, in the wild, zebrafish life expectancy is about two years, but it can reach up to five years under controlled conditions in captivity (Gerhard et al., 2002).

Zebrafish as model organism for toxicological analysis

The aim of toxicology is to determine the safety and effectiveness of possible new drug candidates (Nass et al., 2018). In this perspective, evaluations on biological systems, *in vitro* and/or *in vivo*, are needed to reveal the species-, organ- and dose-specific effects of the substances under investigation (Parasuraman, 2011; Cornet et al., 2018). Cell or tissue cultures allow cheap, rapid and high-throughput analysis, but they lack of physiological context, resulting in low predictive power of toxicity. On the other hand, experimentation on model organisms evolutionarily close to humans is long and expensive, and heavily restricted by country- and agency-specific ethical standards (Ali et al., 2011; Truong et al., 2011; He et al., 2014). The use of zebrafish as model organism for toxicological analysis overcomes these limitations by combining the power of whole-animal investigations with small resources requirement comparable to those of cell culturing (Truong et al., 2014; Nishimura et al., 2015; Cornet et al., 2018).

The most advantageous aspects of this animal model arise from the early developmental stages. Fertilization is external and embryos maintain their transparency until the larval stage, thus facilitating detailed evaluation of physiological structures and organ systems. Moreover, embryo development is very rapid, thereby sensibly reducing the experimentation time (Glass & Dahm, 2004; Veldman & Lin, 2008). The chorion is permeable to a range of small molecules, allowing administration of the compounds of interest directly into the culturing medium. Furthermore, owing to their small size, zebrafish embryos can be individually placed in multi-well plates, thereby limiting the amount of materials required per experiment (He et al., 2014; Truong et al., 2014).

Other aspects of zebrafish biology further avail their usefulness as model organism for screening and testing. Notably, zebrafish have high fecundity and fast life cycle. Once they reach sexual maturity, females are able to spawn several hundred eggs weekly. Such large offspring sizes provide an ideal platform for high-throughput assays with high statistical power (Veldman & Lin, 2008; Arunachalam et al., 2013). Furthermore, captive zebrafish live can breed all year round instead of seasonally, thereby avoiding time limitations on research (Ali et al., 2011; Meyers, 2018).

Besides these biological aspects, zebrafish maintenance is easier and cheaper than mammalian models, thus allowing low cost experimentation.

Additional features support the use of zebrafish as model organism for toxicological assessments. Comparison of the zebrafish to human protein-coding genes revealed not only that 70% of human genes possess at least one zebrafish orthologous, but also that 82% of human morbid genes have a zebrafish counterpart. Zebrafish genome sequencing further revealed a 30% of duplicated genes with

shared function, because of the whole-genome duplication event occurring in the teleost lineage (Howe et al., 2013). Therefore, zebrafish may have two copies of each human orthologue and this enhances analyses of gene regulation (Phillips & Westerfield, 2014).

Toxicity tests in drug development involve the study of absorption, distribution, metabolism and excretion phenomena, collectively referred to as ADME. Since zebrafish embryos and larvae share several physiological, morphological similarities with mammals, they can conveniently substitute mammal models for ADME studies in pharmacology (Pellegatti, 2012; Diekmann & Hill, 2013; Cornet et al., 2018). Moreover, the use of zebrafish as model organism brings direct Replacement, Reduction and Refinement (3Rs) benefits required by the European Directive 2010/63 on the ethical use of animals for scientific purposes. These principles apply to all non-human vertebrates and independently feeding larval forms. Hence, another aspect promoting the use of zebrafish in research is that larvae up to 120hpf do not fall into regulatory frameworks dealing with animal experimentation, because zebrafish start seeking prey and feed independently after 120hpf (Fleming et al., 2007).

The reliability of zebrafish models lies not only in the zebrafish homology to mammalian morphology and biology, but also to behaviour. In particular, both larvae and adult zebrafish display a wide range of complex behaviour patterns, including social, anxiety, learning, memory and defensiveness, closely parallel to mammalian, thus suggesting the evolutionarily conserved nature of many behaviours across species (Kalueff et al., 2014; Stewart et al., 2014). Indeed, despite neuroanatomical differences between mammals and teleosts, homologous functions exist in several key zebrafish brain areas (Randlett et al., 2015; Perathoner et al., 2016; Lucini et al., 2018). In addition, locomotion in zebrafish results from a complex network of evolutionarily conserved pathways and neurotransmitter systems (Grillner et al., 2005; Horzmann et al., 2016). Data collected from larval locomotion are successfully considered for identifying potential central nervous system beneficial and side effects of new drugs, as well as for detecting therapeutic and target specificity of compounds (Kokel et al., 2010; Bruni et al., 2014; Khan et al., 2017; Fontana et al., 2018).

Taken together, the aforementioned factors highlight the versatility and appropriateness of zebrafish as experimental model system for comprehensive large-scale high-throughput chemical screenings, allowing at the same time the minimization of the use of mammalian models without losing reliability (Ali et al., 2011).

Conditioned media

Finding novel therapies is the main goal of toxicity testing for drug development processes. In this context, cell culture-derived conditioned media have been proposed as a promising pharmaceutical candidate for regenerative medicine (Kim and Choi, 2013; Vizoso et al., 2017). Conditioned media are formed during culturing, when cells secrete into the extracellular space many factors, collectively referred to as the secretome, including soluble proteins (e.g. growth factors, chemokines, cytokines), free nucleic acids (e.g. microRNAs), lipids, and different extracellular vesicles. Although a conditioned medium can be harvested from various cell types, the same cells can yield different secretomes depending on the overall cell number, culture duration and conditions (e.g. normoxia/hypoxia, monolayer/spheroid cultures) (Pawitan, 2014; Vizoso et al., 2017).

For almost two decades, mesenchymal stem cells-based therapies have been successfully employed in regenerative medicine (Vizoso et al., 2017). However, the work of Gnecchi et al. in 2005 showed that the paracrine factors released by the cells are responsible for the observed beneficial effects (Gnecchi et al., 2005). Thereafter, several studies on the application of cell-derived secretome in various degenerative diseases revealed the improvement of the pathological conditions after the treatments. It has been demonstrated that mesenchymal stem cell-derived conditioned medium is sufficient to significantly improve multiple biomarkers of pathophysiology, and, in general, to be as effective as transplantation of the corresponding mesenchymal stem cells in several animal models, especially rodents (Vizoso et al., 2017).

Due to their composition, conditioned media may have anti-apoptotic, anti-microbial and anti-tumoral effects. Furthermore, they have a beneficial role in wound healing and tissue repair, having a correlation with the angiogenetic and neurotrophic effects observed (Mishra & Banerjee, 2012; Beer et al., 2017; Vizoso et al., 2017; Sohn et al., 2018). Given the aforementioned evidence, the use of conditioned medium in regenerative medicine is very appealing. In addition, it may offer considerable potential advantages over living cells-based therapies, in terms of manufacturing, storage and handling.

Conditioned media can be easily stored, freeze-dried, packaged and transported without loss of product potency. Furthermore, mass-production of ready-to-use conditioned media is possible through tailor-made cell lines under controlled conditions to obtain the convenient composition for therapeutic applications. In this way, it is possible to reduce the time and cost of expansion and maintenance of cell cultures and to avoid invasive cell collection procedures (Vizoso et al., 2017).

Noteworthy, the adoption of cell-free therapies rather than cell engrafts avoids rejection by the target organism and reduces other related risks, such as immune compatibility, tumorigenicity and the transmission of infections (Pawitan, 2014).

Given all these strengths, cell-derived conditioned media display promising prospects as pharmaceuticals for regenerative medicine. Therefore, conditioned media must be preventively evaluated for safety, efficacy and dosage in a manner analogous to pharmaceutical agents (Kim & Choi, 2013; Vizoso et al., 2017). From this perspective, *in vivo* toxicity assays for these products are essential and zebrafish embryo is the best model organism to use for this purpose. However, literature on zebrafish conditioned media assays is still very limited, mostly relating to studies on adult zebrafish (Cassar et al., 2020).

AIMS

The fulcrum of the work described in this thesis is the use of zebrafish for translational research. Since the zebrafish laboratory recently established at the University of Palermo has been authorized slightly before the start of this PhD project, the preliminary purpose was to improve the maintenance of the fish facility by applying and optimizing protocols for regular housing, feeding, breeding adult fish and handling embryos. In doing so, we ensured the optimal life conditions for zebrafish to obtain viable and sufficient offspring for performing the downstream experiments.

Then, to combine at best both the aims of the PhD course in Technologies and Sciences of Human Health, we organized our work in two main sections: one mostly technological and one purely scientific. In particular, we first devised a multi-parametric assay platform for the *in vivo* toxicological analysis of potential therapeutic compounds and new drug candidates. This assay allows rapid and simultaneous analysis of morphological, apoptotic, behavioural and molecular changes inflicted by multiple compound at different concentrations during zebrafish embryogenesis.

Once the robustness of this pipeline was successfully validated using distinct chemicals known to induce developmental aberrations in zebrafish, we challenged it using complex biological samples. Intriguingly, we show that exposure of zebrafish embryos to conditioned media derived from Wharton's jelly mesenchymal stem cells confers protective effects against apoptosis and oxidative stress.

Altogether, these findings not only provide a novel implementation of current *in vivo* toxicological assays, but also highlight the promising therapeutic potency of conditioned media.

MATERIALS AND METHODS

Facility management and husbandry conditions

Wild type AB strain zebrafish (*Danio rerio*) were housed in the fish facility of the Advanced Technologies Network Center (Aut. n. 06/2017-UT 30/03/2017) of the University of Palermo. All the experiments described in this thesis were performed exclusively on embryos and larvae within 5 days post fertilization (dpf), thus not subject to animal experimentation rules according to European (2010/63/UE) and Italian (D. lgs. 26/2014) directives.

Fish were housed in tanks held in automatic circulating systems Tecniplast – ZebTec ActiveBlue Stand Alone that automatically controls the following parameters:

- Temperature: 28.5°C
- Conductivity: 300 ± 50 μS
- pH: 6.5-7.5

The system was periodically provided with a 0.6% Sodium Bicarbonate (Sigma Aldrich) solution and a 0.6% Instant Ocean® (Aquarium Systems) solution to make adjustments and ensure the right values of pH and conductivity, respectively. Lighting conditions were kept at a fixed 14-10 hours light-dark cycle.

Fish were kept at a maximum density of 5 fishes/L and fed twice a day with live and newly hatched brine shrimps (*Artemia salina*) and with dry flakes (Tetra® TetraMin® Tropical Flakes). *Artemia salina* cysts were placed, at a density of 5g of cysts per liter, in specific aerated brine shrimp hatcher (Hatch-Rite III, Florida Aqua Farms) filled with 3% NaCl osmotic water at 25°C. After 48 hours, the aeration was removed and the hatched nauplii are collected with a brine shrimp sieve. The nauplii were rinsed twice with system water and then fed to the fish with a plastic Pasteur pipette (about 2ml for 5 fish).

Male and female adult fish were preferably housed in separated tanks and set up in pairwise crosses (ratio 1:1) in the afternoon before embryos were required. Male and female were separated through a divider in specific 1 liter-breeding tanks filled with static water. The divider was removed the next morning, shortly after the onset of light, allowing fishes to spawn. The fish were left to mate undisturbed for at least one hour. After breeding, the adults were returned to their tanks in the rack.

Experimental pipeline for toxicological analysis on developing zebrafish

Embryo handling and chemical exposure

The assay was based on the OECD guidelines on Fish Embryo Toxicity (OECD, 2013a; OECD, 2013b). Newly laid embryos were obtained by natural and spontaneous fertilization by coupling males and females in appropriate conditions. Embryos were collected using a strainer, rinsed in E3 medium (Westerfield, 2007; https://zfin.org/zf_info/zfbook/cont.html) added with Methylene Blue (Sigma-Aldrich, CAS no.122965-43-9) and transferred in Petri dishes, labelled with birth date and parental couple tank number. Unfertilized eggs and debris were removed and embryos were incubated in Petri dishes at 28.5°C until staged for following steps.

At 4hpf, synchronous and healthy embryos were selected and gently transferred (1 embryo/well) using a sterile plastic Pasteur pipette into sterile 96 well-plates (Costar 3599, Corning Inc.).
Embryos were never allowed to dry out and were incubated in E3 medium at all times until chemical exposure at 6hpf. The developmental stage was determined according to the description of zebrafish development of Kimmel et al. (Kimmel et al., 1995).
The following compounds were used for protocol set up. The concentrations used are sub-lethal in light of the data of previous studies:

- Cadmium chloride ($CdCl_2$) (Carlo Erba, CAS no. 35658-65-2): 9μM – 25μM – 50μM (Monaco et al., 2017; Capriello et al., 2019)
- Dimethylsulfoxide (Sigma Aldrich, CAS no. 67-68-5): 1% – 1.5% – 2% (Chen et al., 2011)
- Ethanol (Sigma-Aldrich, CAS no. 111-76-2): 1% – 2% (Chen et al., 2011 Ramlan et al., 2017)
- Tricaine (MS-222 Sigma-Aldrich, CAS no: 886-86-2): 50mg/L – 100mg/L – 150mg/L (Félix et al., 2018)

Chemicals of interest were diluted in E3 medium to reach the desired concentrations. E3 medium was removed from each well containing an embryo and replaced with 200μl of either freshly prepared solution. For each concentration, at least 12 embryos were used, while 12 other embryos were for controls simply maintained into E3 medium.

Microinjection

Microinjection needles were prepared by pulling capillary glass tubes (80 millimeters in length, 1/0.8 mm OD/ID millimetre. Cavù s.r.l.) using Narishige Electrode Micropuller (Heater = 8; Magnet = 7.5; Main Magnet = 9.5). The needle was backloaded with 2µl of 0.05 % Phenol Red (Sigma-Aldrich) in E3 medium and then inserted into the needle holder on the Narishige MN-4 Micromanipulator in a proper position to allow for a wide range of movement and adjustment. The tip of the injection needle was opened under the Leica M-205FA Stereomicroscope using Dumont #5 fine forceps to obtain a tip opening of about 20µm. The size of the droplets injected by the needle was calibrated before starting each microinjection session. To do this, the pressure and the time of injection were modulated on the Eppendorf FemtoJet 4i Microinjector by trying several injections in a droplet of mineral oil on a Petri dish. Then, zebrafish larvae were anaesthetized with 0.05% Tricaine (MS-222 Sigma-Aldrich, CAS no: 886-86-2) in E3 medium and positioned on a handcrafted larvae holder (**Fig. 1**), using gel-loading tips to gently shift them around and arrange them as appropriate. Larvae holder was prepared by melting 2% agarose in E3 medium and pouring it in 5 centimetres diameter-Petri dishes embedding two glass capillaries.

Figure 1. *Handcrafted larvae holder. Red arrows point at the capillaries creating the support for larvae.*

Microscopic observation

Live imaging was performed on individual embryos under the Multidimensional Fluorescence Stereomicroscope Leica M205 FA with Leica DFC 550 camera using Leica LAS X Software. Treated embryos and larvae were assessed daily, up to 120hpf, with regard to survival and morphological modifications compared to untreated controls. The larvae were anesthetized with 0.05% Tricaine in E3 medium to prevent movement during the live imaging practice. For a careful examination, the chorion was mechanically removed.

Apoptotic assay

72hpf-larvae were incubated in 2mg/ml Acridine Orange hemi (zinc chloride) salt (Sigma-Aldrich) in E3 medium for 20 minutes. Then, larvae were washed three times in fresh-prepared E3 medium before they were anaesthetized with 0.05% Tricaine (MS-222 Sigma-Aldrich). Stained larvae were protected from light at all times by covering the plate with aluminium foil. Visualization and photographing of apoptotic spots were conducted in a dark room under Multidimensional Fluorescence Stereomicroscope Leica Microsystems M205 FA.

Behavioural analysis

Alive 120hpf-larvae were selected and transferred in a 96 well-plate with one larva per well and 200µl of fresh E3 medium. The plate containing the larvae was positioned inside the ZebraBox observation chamber (ViewPoint Behavior Technologies) equipped with infrared camera. After 10 minutes of acclimation into the chamber, the movement of each zebrafish larva was recorded for 15 minutes without any disturbances. The parameters were set up as follows:

- Colour: black
- Detection threshold: 15
- Movement threshold: Inact/Small = 2 mm/sec. Small/Large = 4 mm/sec
- Time bin: 60 sec
- Light: 50%

The video output from the camera was analysed with the appropriate movement tracking software ViewPoint® ZebraLab Tracking Mode (ViewPoint® Behavior Technologies - version 3.22.3.89). The raw data were processed with ViewPoint® FastData Manager (version 2.4.0.2510) and the charts drawn with Microsoft Excel 2016.

Conditioned medium (CM) exposure

CM derived from WJ-MSC cultures were provided by our collaborators from "*Azienda Ospedaliera Ospedali Riuniti Villa Sofia-Cervello*" of Palermo. The cells were incubated with Dulbecco's Modified Eagle Medium (DMEM) (HiMedia Cell Culture AT068) and cell-free medium (mentioned below as CM) was harvested after 48 hours of conditioning. Then, it was centrifuged to remove cellular debris and the supernatant was concentrated using High-Performance Liquid Chromatography (Lo Iacono et al., 2018).

The CM stock solution had a concentration of 1500µg/mL (referred to as 40X), measured by Bradford protein assay. Starting from 6hpf, zebrafish embryos were exposed to increasing concentrations of CM diluted in E3 medium (1X – 2X – 4X).

Control groups included embryos from the same batches exposed to equivalent volumes of DMEM and freshly prepared Saline Solution (having the same inorganic composition as DMEM: 0.265g/L $CaCl_2 \cdot H_2O$, 0.1 mg/L $Fe(NO)_3 \cdot 9\ H_2O$, 1.98 g/L $MgSO_4 \cdot 7\ H_2O$, 0.4 g/L KCl, 6.4 g/L NaCl) per concentration (1X – 2X – 4X). In parallel, a group of embryos reared in E3 medium was the negative control

Before exposure, a hole was made on zebrafish embryo chorions, whereby ensuring that all the factors contained in the medium could reach the embryo regardless of their size. Zebrafish embryos were exposed to the treatments starting from 6hpf and their development was examined according to the experimental protocol described in this text.

Primer design

Genes of interest were selected based on literature. Information about their gene expression profile were retrieved from ZFIN (https://zfin.org/action/expression/search). Then, NCBI (http://www.ncbi.nlm.nih.gov/Genbank) and Ensembl (http://www.ensembl.org/Danio_rerio) searches in public sequence databases were performed to identify exons and promoter sequences for each selected gene. Primers were designed using the Oligo Explorer Software (version 1.1.2) and further validated on the Oligo Analyzer software (version 1.0.3) to have the following parameters:

- Primer length: 18-22 mer
- Melting temperature: 62°C
- PCR product length: about 150 bp
- ΔG for possible secondary structures: close to 0 kcal/mol

The sequences of each primer were finally checked on the zebrafish genomic reference sequences using Primer Blast (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) to exclude non-specific amplification. The genes considered and the respective primer pair sequences are listed in Appendix A and Appendix B.

Total RNA extraction and reverse-transcription

Total RNA was isolated from 50 30hpf-embryos according to the protocol Purification of Total RNA from Animal Cells using Spin Technology of the RNeasy Mini Kit Qiagen. Before starting, embryos were manually dechorionated and deyolked with Deyolking Buffer. In our hands, about 20µg of total RNA was isolated with this procedure. RNA samples were quantified spectrophotometrically at 260 nm, and the RNA quality was checked by 1.5% agarose gel electrophoresis.

For the synthesis of cDNA, reverse-transcription reactions were carried out following the indications of the TaqMan Reverse Transcription Reagents kit (Applied Biosystems). For each sample, the cDNA was synthesized starting from 2.5µg of total RNA in reactions of 50µl containing 2.5µM random hexamers. The thermal profile was set up as recommended by TaqMan Reverse Transcription Reagents kit (Applied Biosystems):

25°C 10’
37°C 60’
95°C 5’

Chromatin ImmunoPrecipitation (ChIP) assay

ChIP assay was performed by adapting the protocol from Lindeman (Lindeman et al. 2009). Zebrafish embryos at 30hpf were manually dechorionated, transferred in a 1.5 ml tube containing 300µl of deyolking buffer (55 mM NaCl, 1.8 mM KCl, 1.25 $NaHCO_3$) (Link et al., 2006), and incubated for 15 minutes. For each experiment, 100 embryos of each group were used. The embryos were gently pipetted with a narrow tip every 5 minutes to ease yolk dissolution. After centrifugation for 4 minutes at 1100 rpm to remove yolk residuals, the embryos were incubated in 300µl of trypsin solution (Covassin et al., 2006) at 28°C for 20 minutes, during which they were grinded by pipetting every 10 minutes. At the same time, digestion trend was monitored by checking 2µl of solution under Leica DMi8 Inverted Microscope. Trypsin was blocked by adding 1mM $CaCl_2$ and 10% fetal bovine serum. After centrifugation and supernatant removal, cells were rinsed twice in ice cold PBS 1X. Dry cells were immediately cross-linked by adding 1% formaldehyde in PBS 1X and incubated for 8 minutes.

To quench formaldehyde, 0.125 M glycine was added and the cells were incubated for 5 minutes. After supernatant discard, the cells were resuspended in a solution of ice cold PBS 1X with 1:100 PMSF and 1:100 Protease Inhibitor Mix (Sigma-Aldrich). Cell pellet was washed three times with this solution and two times with ice cold PBS 1X.

Cell pellets were resuspended in 150µl of ice-cold Lysis Buffer (50mM Tris HCl pH 8, 10mM EDTA, 1% SDS, 1:100 PMSF, 1:100 Protease Inhibitor Mix). Sonication was carried out in a high-power ultrasonic bath (Sonorex Digitec DT 103 H, Bandelin) at variable cycle numbers between 15 and 105 (1 cycle = 30s ON/ 30s OFF) to reach the optimal average fragment size of 250bp, as determined by 2% agarose gel electrophoresis. After sonication, the chromatin was recovered in clean tubes by centrifugation.

In parallel, antibody-bead complexes were prepared. Protein A-sepharose magnetic beads (Protein A Mag SepharoseTM Xtra- GE Healthcare) were washed twice with 2.5 V of RIPA buffer (10mM Tris HCl pH 7.5, 140mM NaCl, 1mM EDTA, 0.5mM EGTA, 1% Triton X-100, 0.1% SDS, 0.1% Na-deoxycholate). 10µl of magnetic beads slurry per each ChIP reaction were loaded in different tubes and incubated with 2.4µg of titrated amount of antibody overnight at 40 rpm at 4°C. Aliquots of slurry were incubated in the absence of antibody, as negative control.

Afterwards, 1µg of sonicated chromatin was added for each ChIP reaction and negative control, and incubated for 2 hours at 40 rpm at 4°C. In parallel, an equivalent amount of cross-linked chromatin (Input) was withdrawn and processed as the immunoprecipitated chromatin.

The immune-complexes were adsorbed to protein A-sepharose beads, which were sequentially washed twice with ice-cold RIPA and once with TE Buffer (10mM Tris HCl pH 8, 10mM EDTA). The immune-complexes were eluted with ChIP Elution Buffer (20mM Tris HCl pH 7.5, 5mM EDTA, 50mM NaCl, 1% SDS, 50µg/mL Proteinase K) and incubated at 1300 rpm at 68°C for 2 hours to reverse the cross-linking.

DNA from chromatin samples was extracted with phenol-chloroform and precipitated with absolute ethanol, sodium acetate 0.3 M and glycogen overnight at -80°C. Finally, DNA was dissolved in 50µl of MilliQ water. DNA samples were then quantified by readings in a Qubit Fluorometer (Invitrogen) using the Quant-iT dsDNA HS Assay Kit (Invitrogen).

Polymerase Chain Reaction

Expression level of the genes of interest, as well as the enrichment of histone regulatory sequences in genomic DNA purified from the precipitated chromatin fractions, were examined by semi-quantitative PCR, whose conditions were as follows:

94°C	10''	⎫
58°C	30''	⎬ 45 cycles
72°C	30''	⎭
72°C	2'	

Amplification primer pairs for each target are listed in Appendix A for cDNA analysis and Appendix B for ChIP. Quantification of PCR products was performed on 2% agarose gel electrophoresis using Molecular Imager® VersaDoc™ Bio-Rad and Quantity One® Bio-Rad software.

RESULTS

Set up of the facility management and husbandry conditions

Current zebrafish husbandry conditions derives from knowledge of zebrafish natural habitat along with recorded experiences of maintaining in laboratory conditions (Harper & Lawrence, 2011; Reed & Jennings, 2011). Welfare and successful breeding are the main aspects to consider when handling zebrafish for scientific research. Therefore, standards and conventional approaches for zebrafish caring and management have been established over time from the perspective of breeding this species for scientific purposes (Garcia et al., 2016; Lawrence et al., 2016; Tsang et al., 2017).

Since the zebrafish laboratory has been very recently established at the University of Palermo, we first optimized the facility management. In particular, we standardized all the daily operations and procedures according to the most recent guidelines and current law for animal welfare in scientific research (D. Lgs 26/2014, Dir. 2010/63/EU), in order to maintain healthy and successfully breeding adults (Westerfield, 2007; Reed & Jennings, 2011; Avdesh et al., 2012; Guillen et al., 2012; Geisler et al., 2016).

Zebrafish are relatively tough animals, which easily tolerate a wide range of environmental parameters in the wild. However, in captivity zebrafish are kept under conditions that mimic the monsoon season, when breeding naturally occurs (Reed & Jennings, 2011). In our facility zebrafish are housed in tanks equipped by systems that continuously filter, aerate and disinfect water, guaranteeing automatic monitoring of the optimal water parameters chosen.

The biological clock of the zebrafish is modulated by the photoperiod. In the wild, zebrafish experience around 10-12 hours of darkness per day. In the laboratory, setting a proper photoperiod replicating the circadian light cycle has a significant influence on the mating behaviour, whereas fish spawn early in the morning. Along this line, we set up an automatic photoperiod of 14 hours of light (from 9 a.m. to 11 p.m.) and 10 hours of darkness per day, as widely recommended (Westerfield, 2007; Reed & Jennings, 2011; Tsang et al., 2017).

The next critical step was to keep optimal temperature. Although zebrafish can survive temperatures from 6°C to 38°C in their natural habitat (Reed & Jennings, 2011), it is well-known that they grow and breed satisfactorily at a water temperature of 28.5°C (Kimmel et al., 1995; Westerfield, 2007; Tsang et al., 2017). For this reason, in our facility the water temperature is maintained at this value, while room temperature is set up about 2°C lower to guarantee a more comfortable working environment to operators (Avdesh et al., 2012).

A fundamental parameter to consider for preserving zebrafish health is water salinity. In nature, zebrafish adapt to a variety of salinity conditions, ranging between 10 and 1500µS (Avdesh et al., 2012). Facilities worldwide set up salinity level on their own within this range, depending on practical issues. In our facility, we tested two ranges of salinity: 600µS - 400µS, with optimum at 500µS; and 400µS-200µS, with optimum at 300µS. Setting up the first salinity range leads to huge salt accumulation and, consequently, the risk of corrosion essential mechanical parts of the system. By contrast, we observed that the lower salinity range minimizes salt accumulation. Moreover, we verified that setting salinity around 300µS does not affect fish viability. Indeed, this condition replicates the dilution of water occurring in nature during breeding season, characterized by heavy rain (Reed & Jennings, 2011). This evidence has lead us to set the salinity around 300µS. The system automatically controls the salinity value and makes adjustment as needed by adding a solution of a commercial salt mixture specific for aquaculture.

The pH is essential to maintain a good water environment. In nature, zebrafish live in water with a pH ranging from 5.9 to 8.1 (Reeds & Jennings, 2011). The pH of housing water in aquatic systems may have significant effects on zebrafish health. First of all, a pH level at or close to neutral allows the survival of beneficial nitrifying bacteria living in the biological filters of the systems. These beneficial bacteria metabolize the nitrogenous waste excreted by fish, as they oxidize ammonia to nitrite and subsequently to the much less toxic nitrate. This process acidifies water, thus reducing the risk of proliferation of other microbes, which can be harmful to fish and cause infections (Geisler et al., 2016; Tsang et al, 2017). Our system is provided with a sodium bicarbonate solution, which is introduced as needed into the circulating water to maintain the pH between 7 and 8.

Water quality depends also on population density at which fish are kept inside the tanks. Zebrafish are shoaling fish that prefer to live in group and have small sizes, as their body length is only about four centimetres. For these reasons, it is possible to house efficiently a large number of individuals in small spaces. However, over-crowded population density is detrimental for zebrafish welfare. (Westerfield, 2007; Tsang et al., 2017; Aleström et al., 2019). Indeed, the amount of ammonia excreted by fish may become too much to be neutralized by the metabolic activity of nitrifying bacteria, thus making zebrafish prone to infections. On the other hand, keeping fish alone may provoke stress and suffering. In our facility, we housed zebrafish in small groups, at a maximum density of 5 fishes/L, as widely recommended (Guillen, 2012).

We generally prefer to separate fish in tanks by gender. This avoided casual spawning, aggressive behaviour and hierarchies, but also allowed easy distinction of male and female individuals for

mating. However, we allow fish to breed every week, safeguarding their welfare and their natural behaviour.

Successful breeding is strictly related to an equilibrate diet (Watts et al., 2016). Zebrafish are omnivorous: in the wild, their diet consists of zooplankton, fitoplankton, algae, spores and invertebrate eggs (Spence et al., 2007). Several commercial dry aquarium flakes are available to provide the proper nutritional intake to fish. We made a careful selection to choose the best flakes in terms of value for money. We also supplement dry food with live *Artemia salina* nauplii. Nauplii are fed to fish at 24 hours after hatching, because at this stage they have a good content of proteins, essential fatty acids and vitamins (Westerfield, 2007). It is widely accepted that a combination of dry and live feeds improves survival, growth and reproductive performance (Varga et al., 2016). Moreover, the swimming behaviour of nauplii stimulates fish natural hunting instinct, thus providing an important environmental enrichment (Hoo et al., 2016; Wafer et al., 2016).

Mating is the crucial aspect to consider in zebrafish husbandry for scientific purposes. Indeed, obtaining enough offspring is the first challenge for successful experiments. We improved our mating protocol by applying suggestions from papers (Westerfield, 2007; Engezer et al., 2007; Nasiadka & Clark, 2012; Hoo et al., 2016) and courses. In this way, we witnessed an increase of successful mating from 40% to 80% in one year.

In nature, zebrafish mate in slow-flowing and shallow waters. For this reason, mating couples are placed in commercial special designed flat and shallow plastic tanks (**Fig. 2**). The mesh at the bottom prevent fish from eating sinking eggs.

Figure 2. *Female (left side) and male (right side) adult zebrafish in mating tank. Red arrow indicates the divider, while black arrow the mesh at the bottom.*

Zebrafish spawn early in the morning, immediately after the onset of the light. Therefore, the evening before embryos are needed, breeding fish were set up, preferably away from meals to obtain clean egg clutches without excrements. Since the holding tanks are blue, we usually place the transparent breeding tanks on blue supports, so that the fish feel at ease and they are involved to spawn. We observed that this trick has often worked. When synchronous embryos are required, males and females are separated inside the tanks by a divider, which is removed in the morning to allow sexual courtship and spawning at a specific time. Otherwise, no divider is inserted and fish are free to spawn at any time.

Set up of an experimental pipeline for toxicological analysis on developing zebrafish

Once basic conditions for zebrafish husbandry and breeding have been set up, we devised a multi-parametric embryo-larval experimental workflow to evaluate the developmental toxicity of new potential therapeutic compounds. The procedure involves the following steps:

1. Exposure of embryos to specific substances
    1a. Microinjection of specific substances
2. Microscopic observation of developing embryos
3. Apoptotic assay on 72hpf embryos
4. Behavioural analysis of swimming larvae
5. Molecular analysis: gene expression and epigenetic analysis.

1. Exposure of embryos to specific substances

Embryo treatments were conducted in 96-well plates at a density of 1 embryo per well. We started the chemical exposure from 6hpf, the so-called Shield stage, when the maternal-to-zygotic transition of gene expression is almost complete (Stehr et al, 2006; McCollum et al., 2011). To guarantee statistical significance, we used groups of 12 embryos per each treatment condition and untreated controls and each experiment was repeated with different batches of embryos. Synchronous sibling embryos were used in each experiment to avoid any bias in the results due to different developmental stage or genetic condition. In a preliminary phase, we assessed different concentrations of chemicals

known for their potential toxic effects on developing zebrafish, as detailed in the Materials and Methods section.

1a. Microinjection of specific substances

We have decided to further refine our pipeline by including microinjection, a widely used technique in experimental biology and toxicological analysis (**Fig. 3**), to deliver specific substances into selected areas of developing zebrafish. Unlike the classical Fish Embryo Toxicity Test (OECD, 2013a; OECD, 2013b), microinjection allows direct administration of both polar and non-polar substances, low and high molecular size compounds or even cells, overcoming natural barriers such as the chorion (Schubert et al., 2014; Samaee et al., 2017).

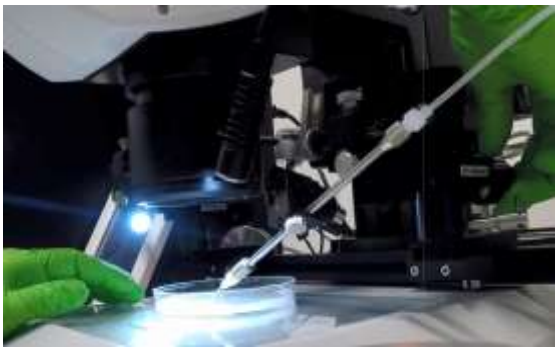

Figure 3. *Preparing the microinjection apparatus under the stereomicroscope before starting session.*

In particular, we were interested in applying this technique for testing potential therapeutic compounds and drug carriers targeted for the treatment of retinitis pigmentosa (RP), one of the most common retinal degenerative disorders. Intravitreal injection is generally considered as the most effective way of delivering materials to the back of the eye, where the retina is located. In addition, we pointed out that zebrafish is an advantageous model, as fish eye is structurally similar to that of mammals (Giannaccini et al., 2014; Angueyra and Kindt, 2018). Our purpose was to evaluate the ability of intravitreally-injected drug candidates or differentiated mesenchymal stem cells to rescue retinal defects either in wild type zebrafish having chemical-induced retinal degeneration or in larvae obtained from specific RP transgenic lines.

In parallel, we practiced intravenous microinjection, an excellent tool for the introduction of a variety of soluble substances into the bloodstream of zebrafish larvae to reach their target areas. For instance,

this technique can be employed to test the efficiency and the potential side effects of gene therapy and drug delivery carriers (Cianciolo Cosentino, 2010; Duan et al., 2016).

We have carried out several tests using different injection volumes to assess the best conditions for our needs. The injection volume was calculated by comparing it to the drop size based on the conversion table from Cold Spring Harbor Protocols (**Tab. 1**).

Diameter of drop (µm)	**Radius of drop (µm)**	**Volume ($4/3[\pi r^3]$) (nl)**
150	75	1.77
160	80	2.15
170	85	2.58
180	90	3.06
200	100	4.20
225	112.5	5.90

Table 1 *Conversion table for calculating microinjection volumes (http://cshprotocols.cshlp.org/content/2010/12/pdb.tab195537).*

Finally, we considered that a volume of about 2-3nl was the optimum for intravitreal injections, while the injection volume could be increased to 5nl for bloodstream injections (Cianciolo Cosentino et al., 2010; Giannaccini et al., 2014).

Zebrafish larvae were generally injected at 48 or 72hpf, when the development of retinal structure has already started and the circulatory system is complete. Moreover, the yolk sac was reduced, so positioning the larvae on the plate for the microinjection was easier. Successfully injected larvae were identified by observing the fluorescence in the vasculature or in the eye immediately after injection (**Fig. 4**).

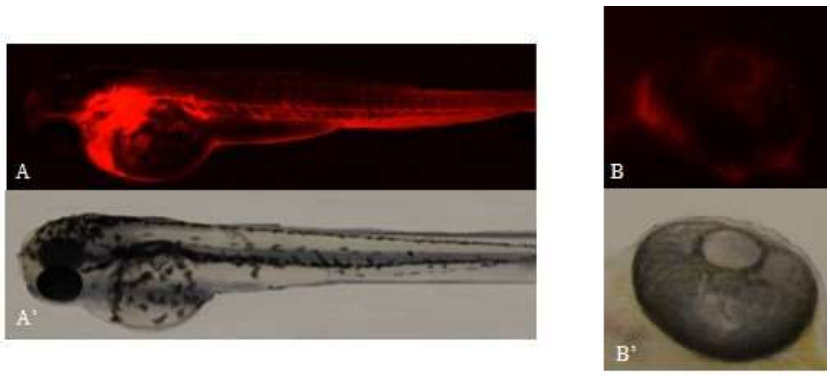

Figure 4. *Representative examples of injected 48hpf-larvae with the Texas Red-conjugated dextran fluorescent dye in the bloodstream* ***(A)*** *and in the retinal epithelium* ***(B). A'*** *and* ***B'*** *are the equivalent pictures in bright field.*

After acquiring these skills, I moved to the Department of Molecular Life Sciences of the University of Zurich, where I should have used the described techniques for the following experiments of my thesis. The original plan involved the trans-differentiation of WJ-MSCs, derived from human umbilical cord discarded at birth, into photoreceptor-like and retinal pigment epithelium-like cells. Then, we would have evaluate the different ability of naïve and differentiated WJ-MSCs to rescue retinal defects either in wild type zebrafish larvae treated with N-methyl-N-nitrosourea to induce retinal degeneration or in larvae obtained from appropriate RP zebrafish lines, the latter housed at the host laboratory directed by Professor Stephan Neuhauss. In parallel, we would have evaluate the efficacy of intravitreally-injected morpholino oligonucleotides directed against selected aberrant mRNA splicing variants, for the correction of splicing defects in appropriate RP zebrafish larvae supplied by our colleagues in Zurich.

The degree of vision correction in injected larvae would be assessed by measuring of optokinetic nystagmus and optomotor responses by using specific equipment at the host laboratory. Moreover, human gene expression should have been determined by quantitative PCR in cDNA samples derived from dissected eyes of injected fish.

Unfortunately, the well-known Covid-19 outbreak emergency prevented my work in the laboratory abroad, forcing myself to return in Italy. To make matters worse, the "*Azienda Ospedaliera Ospedali Riuniti Villa Sofia – Cervello"* became a Covid Hospital, hindering our collaborators to provide naïve and differentiated WJ-MSCs. For all these reasons, the related experimental part has been cancelled.

2. Microscopic observation of developing embryos

We investigated the effects of the administered chemicals by checking embryonic development progression. For this purpose, embryos and larvae were observed daily under the stereomicroscope until 120hpf for assessing the survival rate of each treated group and normalizing it on the survival observed in control groups. In parallel, at each time-point, treated embryos and unperturbed controls were examined in detail to identify possible phenotypic alterations induced by the treatments. Somite formation, tail detachment and head development were evaluated at 24hpf, while the presence of heartbeat, oedema and pigmentation was evaluated at 48hpf. After hatching, the absorption of the yolk, the onset of skeletal deformities and variations in the body length were assessed.

Fig. 5 shows representative examples of the observations made during protocol set up.

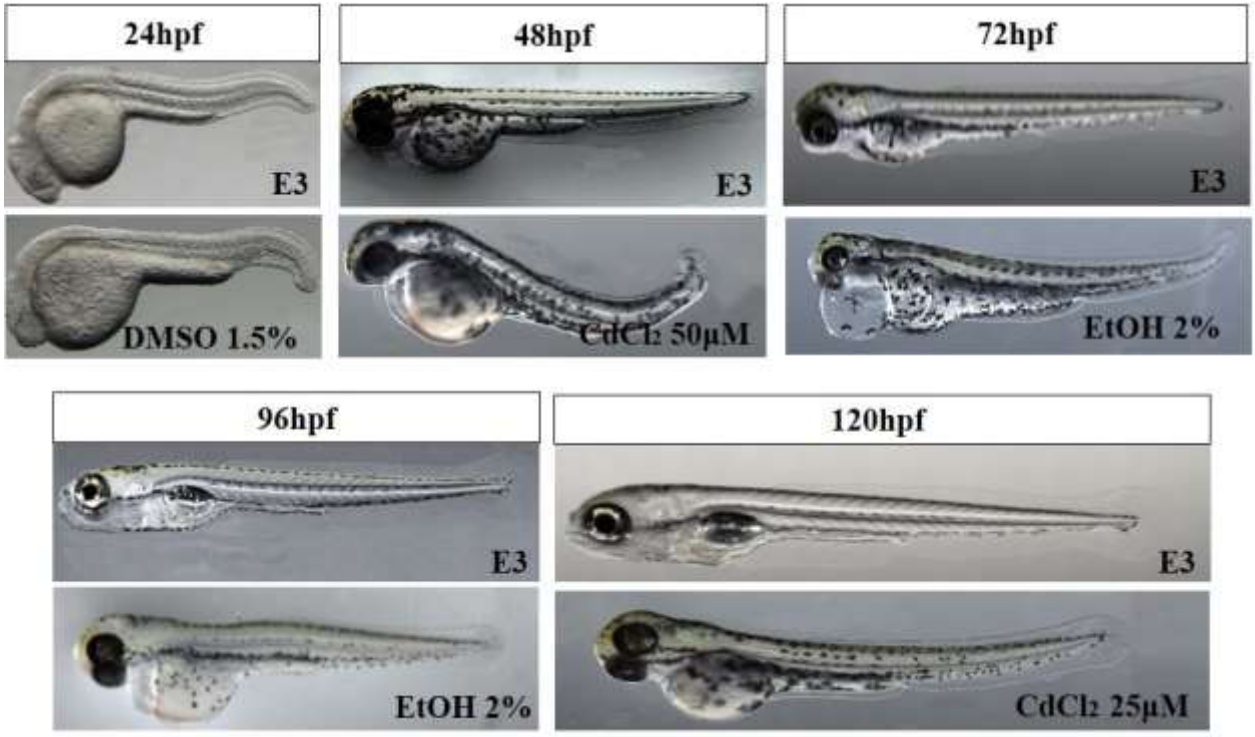

Figure 5. *Representative examples of toxic effects inflicted by exposure to the indicated concentrations of the chemicals used.*

3. Apoptotic assay on 72hpf embryos

Selective labelling of cells undergoing apoptosis was achieved by staining developing zebrafish with the vital fluorescent dye acridine orange (Tucker & Lardelli, 2007). Indeed, this dye permeates cells and emits a particularly strong green fluorescence when intercalated into fragmented DNA, which is a result of the apoptosis machinery at work (Negron et al., 2004; Eimon et al., 2010).

Based on several attempts, we decided to perform this assay at 72hpf because at prior stages numerous foci of physiological morphogenetic and histogenetic apoptosis were detected, making it difficult to visualize apoptotic variations (Cole & Ross, 2001). Moreover, the apoptotic analysis in embryos at 72hpf is convenient due to the reduced background noise derived from the auto-fluorescence of the yolk-sac, which is instead particularly prominent in the earlier stages.

The **Fig. 6** reports a summary of the observations carried out during preliminary tests. In particular, $CdCl_2$-treated larvae display a broad staining pattern throughout the body, underling an enhanced ectopic apoptosis. By contrast, although the larva exposed to DMSO 2% has an abnormal morphology, it shows the physiological apoptotic focus occurring during the development, as shown in the control.

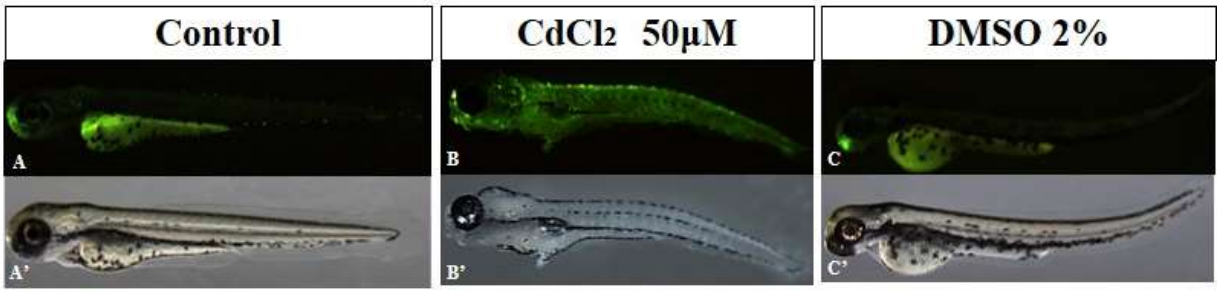

Figure 6. *Acridine Orange staining allows to observe apoptosis in vivo in unperturbed control* ***(A),*** *and in larvae treated with* $CdCl_2$ ***(B)*** *and DMSO* ***(C)****.* ***A', B'*** *and* ***C'*** *are the equivalent pictures in bright field.*

4. Behavioural analysis of swimming larvae

At 120hpf, zebrafish larvae show mature swimming activity, which could provide important information for toxicological assays. Indeed, treatment of zebrafish larvae with potential neuroactive substances could change the response pattern of the larvae, which could then be used to determine whether a substance has a neuroactive property or not. Indeed, these locomotor activities depend on the integrity of brain function, nervous system development, and visual pathways (Ali et al., 2012).
In light of this, we have included behavioural analysis in our protocol. We examined the locomotor activity and movement pattern of zebrafish larvae using the ViewPoint® ZebraLab, an automated live video tracking system (**Fig. 7A**).
Parameters were adjusted as detailed in the Material and Methods section. While the experiment is running, the software detects the trajectories moved by the larvae and marks them by different colours based on the swim speed (**Fig. 7B**). Hence, the first output to evaluate at the end of the assay was the global path image, visually displaying how the larvae moved throughout the experiment (**Fig. 7C**). In parallel, larvae locomotor parameters are quantified by the software from the video recorded during the assay, and these raw data are essential to calculate the mean velocity, which indicates the average speed, measured in mm/sec, of treated larvae compared with that of control larvae (**Fig. 7D**).

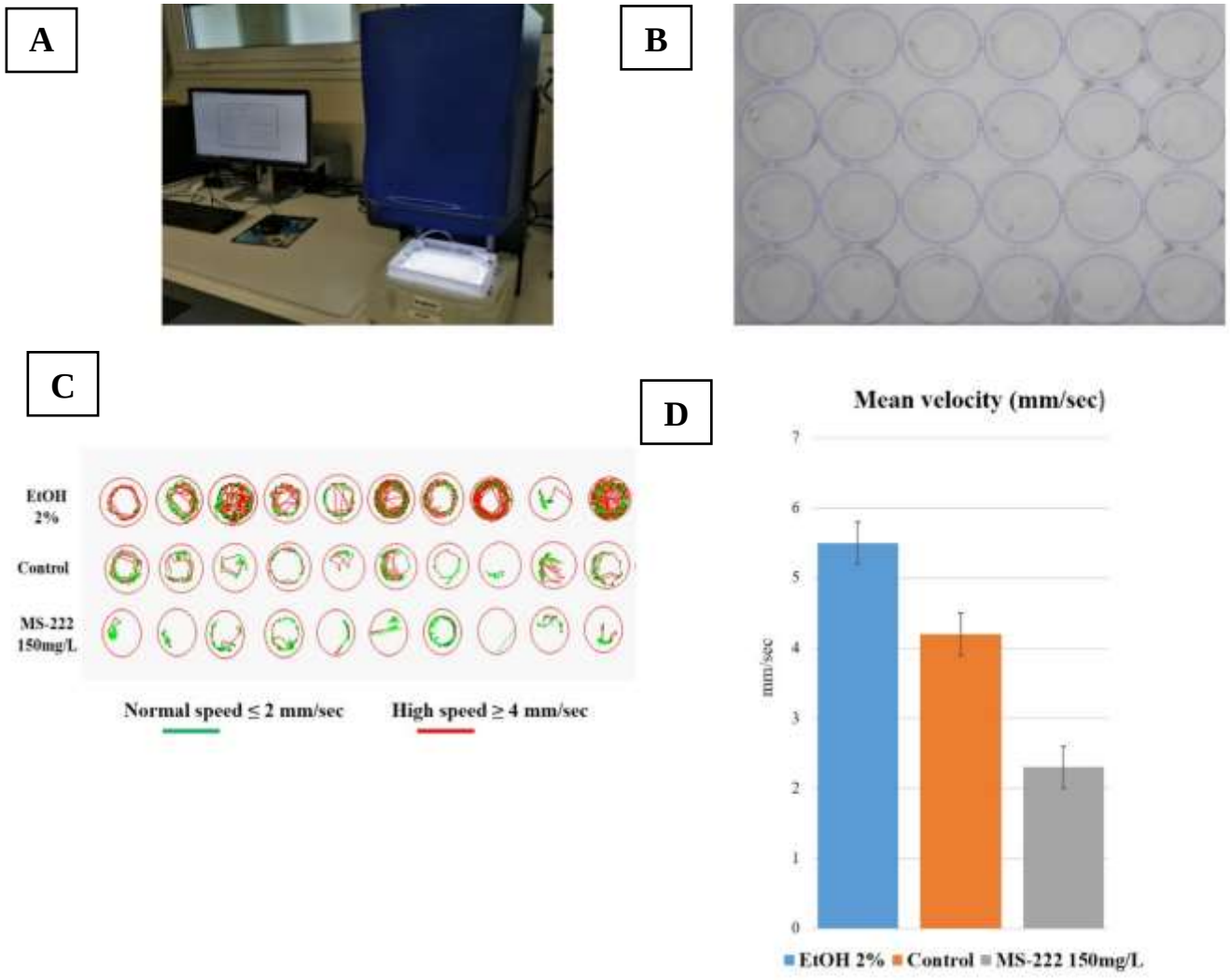

Figure 7. **(A)** *Setting up ViewPoint® ZebraLab for the experiment. Once the plate with larvae is placed and the light switched on, the ViewPoint® ZebraBox chamber is closed and the experiment can be launched.* **(B)** *Frame of a video recorded while the experiment was running. The blue circles mark the wells containing larvae. Coloured lines highlight the trajectories moved by the larvae.* **(C)** *Global path moved by the 120hpf-larvae during the behavioural assay. The colours of the lines refer to different speed maintained, as described in the legend.* **(D)** *Variation of the mean velocity among the considered groups of larvae exposed to different treatments and compared to control unperturbed larvae.*

5. Molecular analysis: gene expression and epigenetic analysis

Embryonic development is driven by changes in gene expression, whereas alteration in the epigenetic landscape and chromatin structure are major mechanisms that can simultaneously activate and repress the expression of multiple genes. Epigenetic marks, such as histone post-translational modifications and DNA methylation, alter nucleosome positioning, chromatin compaction, and transcription factor access to DNA. It is now clear that epigenetic mechanisms collaborate with the underlying genomic information to dictate whether a gene is transcriptionally active or silenced.

In addition, epigenetic mechanisms may specifically mediate toxicity responses to certain chemicals (Baccarelli & Bollati, 2009; Mudbhary & Sadler, 2011). In this regard, we considered worthwhile to introduce in our protocol Reverse Transcription-PCR and Chromatin Immunoprecipitation (ChIP) to identify possible gene-specific transcriptional and epigenetic changes induced by the treatments of interest. While specific gene expression has been easily assessed starting from total RNA samples extracted from fish embryos using commercial kits, a challenging step of ChIP procedure was chromatin preparation. Indeed, ChIP is an experimental procedure *in vivo* that uses antibodies to determine whether a given protein binds specific tracts within a population of fragmented chromatin (Lindeman, 2009; Cavalieri & Spinelli, 2017; Yao et al., 2017; Cavalieri, 2020; Reina and Cavalieri, 2020).

We first tried to disaggregate whole fish embryos into single cells by forcing them through a syringe needle. However, we realized that this procedure was not completely successful and was unsatisfactory in terms of chromatin yield. Therefore, we decided to disaggregate embryos by gentle trypsinization after yolk removal with a specific buffer (Covassin et al., 2006; Link et al., 2006). We have tested that an incubation time of 20 minutes is enough to obtain a sufficient level of embryo dissociation in free intact cells or small aggregates (**Fig. 8**).

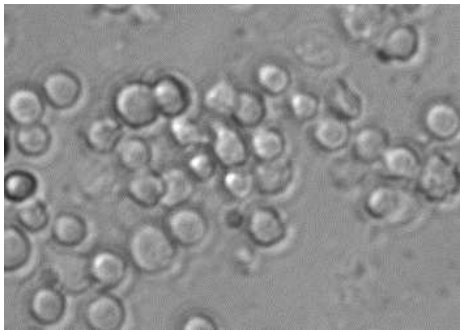

Figure 8. *Single cells suspension derived from embryos trypsinization. (HP PL Fluotar L 40X/0.6 Dry – Leica Microsystems DMi8).*

Cells were then cross-linked with 1% formaldehyde and lysed, as described in Materials and Methods. This procedure typically yield 10µg of chromatin from 100 30hpf-embryos, which was enough for downstream experiments.

Next, we have set up chromatin shearing by testing different sonication times to obtain a population of fragments with an average length of about 250 bp (Sadeh et al., 2016) (**Fig. 9**). This size is close to that of the single nucleosomal fraction, allowing using antibodies against post-translationally modified histones associated to the transcription status of the genes of interest. Finally, genomic sequences associated with the precipitated nucleoprotein complexes were identified by PCR, using specific primer pairs.

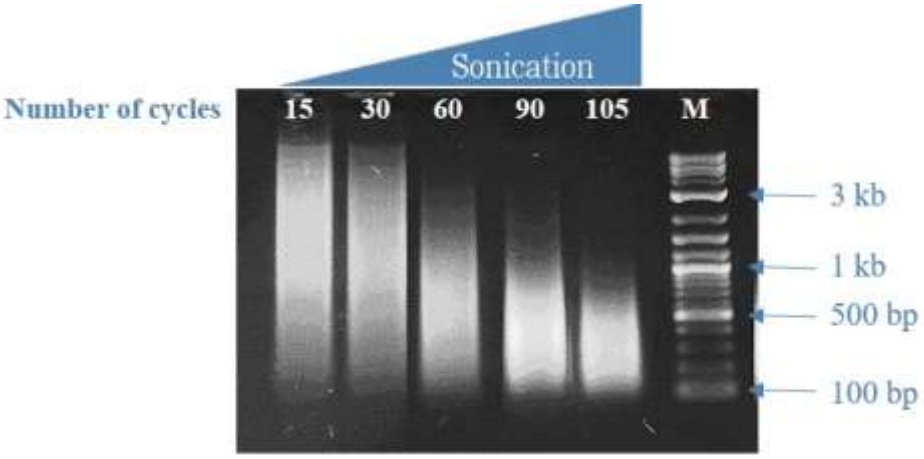

Figure 9. *Electrophoretic migration of reverted chromatin extracted from 30hpf-fixed embryos sheared at different sonication times (1 cycle= 30s ON/30s OFF) and analysed on 2% agarose gel. M = 2 log ladder (New England BioLabs).*

Application of the experimental pipeline for toxicological analysis: developmental effects of exposure to Conditioned Media

We extended the application of each step of our experimental protocol by testing the developmental effects of a complex biological sample, in particular the conditioned medium derived from WJ-MSC isolated by umbilical cord and provided by our collaborators from "*Azienda Ospedaliera Ospedali Riuniti Villa Sofia – Cervello*" of Palermo.

Bradford protein assay of CM revealed a concentration of 1500μg/mL (conventionally referred to as 40X). This stock solution was diluted 1:40 (1X), 1:20 (2X) and 1:10 (4X) in E3 medium. In each assay, embryos from the same batches were used to establish three distinct control groups. Embryos of a first group were reared in the presence of equivalent amounts of DMEM. A second group of embryos was exposed to a "saline solution" having the same inorganic salts composition as DMEM. Finally, a third group of embryos was reared in E3 medium as a negative control.

Thus, embryos were observed every 24 hours until the 120hpf stage, evaluating the survival rate and phenotypic changes, and data presented as the mean of quadruplicate experiments are summarised in **Fig. 10**. As expected, control embryos grown in E3 medium and in the presence of the saline solution were all alive and phenotypically normal at 120hpf. Surprisingly, a significantly reduced survival rate was detected during development of embryos exposed to DMEM. This effect was dose-dependent, causing the premature death of all embryos at 96hpf at the highest concentration of DMEM. By contrast, exposure to CM at all concentrations used did not affect the survival rate until 72hpf. However, at later time-points a dose -dependent decrease in the survival rate was detected in the CM-treated embryos, leading to a maximum of 45% of dead larvae at 120hpf detected at the highest concentration.

As predictable from the survival assessment, we observed the peculiar ability of DMEM to inflict major phenotypic deformities starting from early developmental stages (**Fig. 11**). Indeed, DMEM caused a range of alterations, including severe developmental delay, malabsorption of the yolk, crooked body and onset of pericardial and yolk-sac oedema. These abnormalities arouse in a dose-dependent manner, whereas no normal embryos were detected starting from 24hpf following exposure to the highest concentration of DMEM (**Fig. 10** and **11**). By contrast, exposure to CM generally produced milder phenotypic alterations, whereas the developmental progression of embryos exposed to the highest concentration of CM was not affected and no pericardial/yolk-sac oedema was

observed. In fact, about 25% of these embryos displayed either slight developmental delay or spinal curvature compared to control unperturbed embryos (**Fig. 10** and **11**).

Altogether, these findings suggest that the molecular components contained in CM could somehow shield the toxic effect provoked by DMEM.

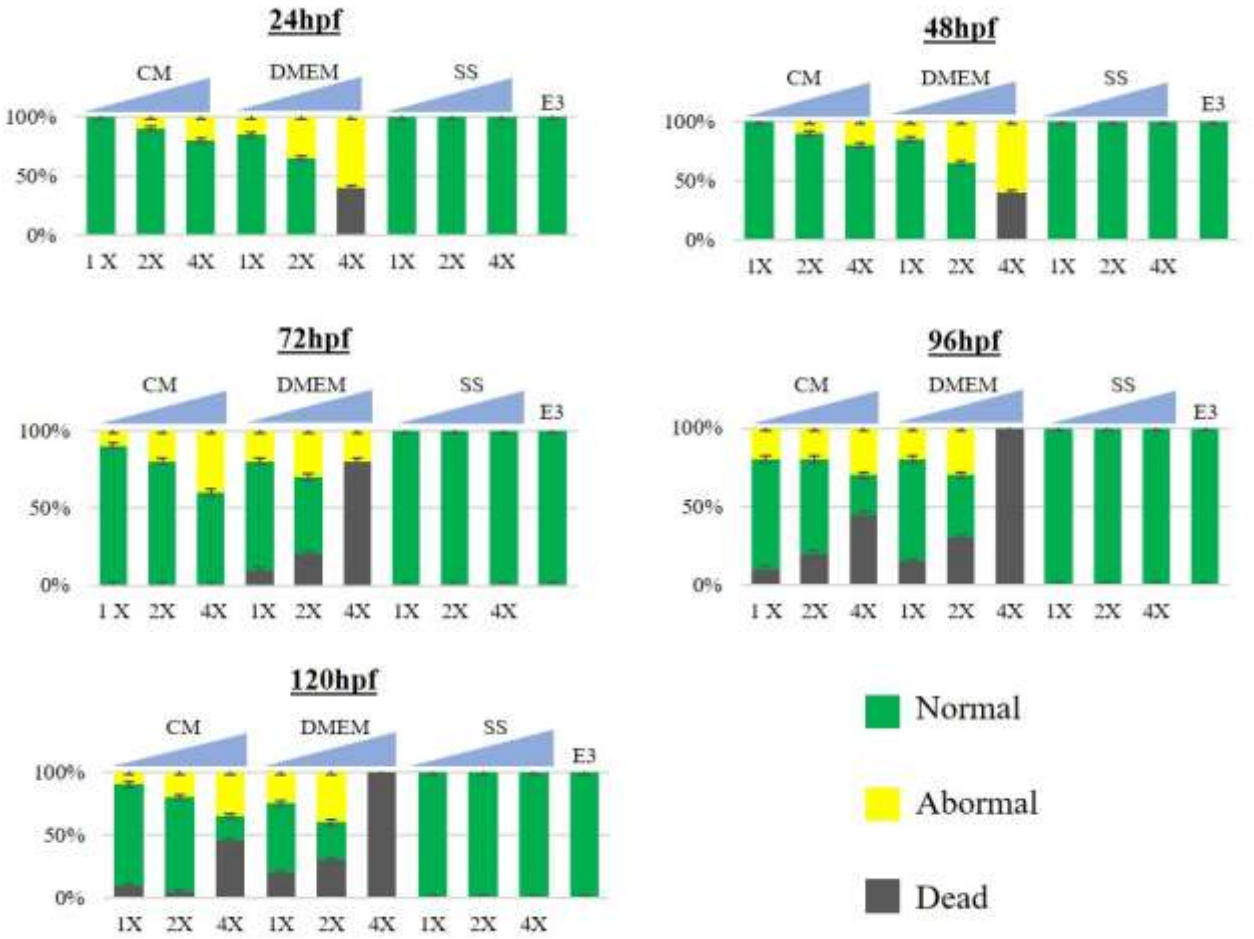

Figure 10. *Survival rate and incidence of abnormal phenotypes of developing zebrafish exposed to the experimental concentrations of CM, DMEM, Saline Solution (SS) and E3 medium. Data are presented as the mean of quadruplicate experiments (10 embryos for each sample). Error bar = ± SD.*

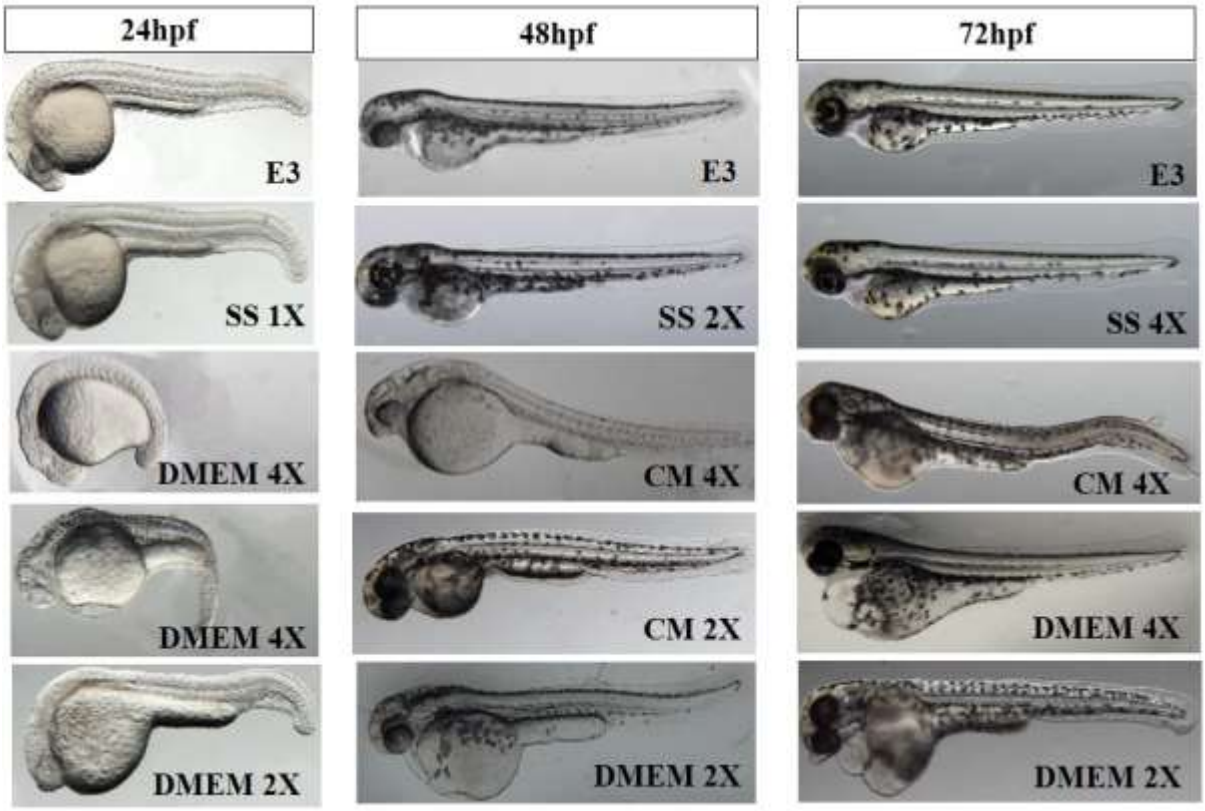

Figure 11. *Representative examples of the phenotypes observed with the indicated treatments.*

Next, by staining with acridine orange we investigated the apoptotic landscape in live control and treated 72hpf-larvae. Besides the above-mentioned control groups, in this assay we also included sibling larvae exposed to the sub-lethal concentration of 9μM $CdCl_2$, which is a potent apoptotic inducer and represents a compelling positive control. $CdCl_2$ was administered 24 hours before the assay started, in order to preserve larvae from drastic damages caused by prolonged exposure (Chan & Cheng, 2003; Monaco et al., 2017; Tucker & Lardelli, 2007; Chiarelli et al., 2019). As expected, control embryos reared in E3 medium and in saline solution displayed the normal developmental apoptotic spatial pattern, including a single greater focal point at the olfactory epithelial cells and a number of smaller apoptotic foci along the tail. Intriguingly, DMEM provoked a dose-dependent appearance of ectopic apoptosis in the pericardial region of the 90% of treated larvae (**Fig. 12B**) highlighting a linear correlation with the incidence of pericardial oedema in these embryos. In striking contrast, larvae exposed to all the concentrations of CM exhibited an essentially normal acridine orange staining, indicating that CM exposure did not induce variation in the physiological apoptotic pattern (**Fig**. **12A**).

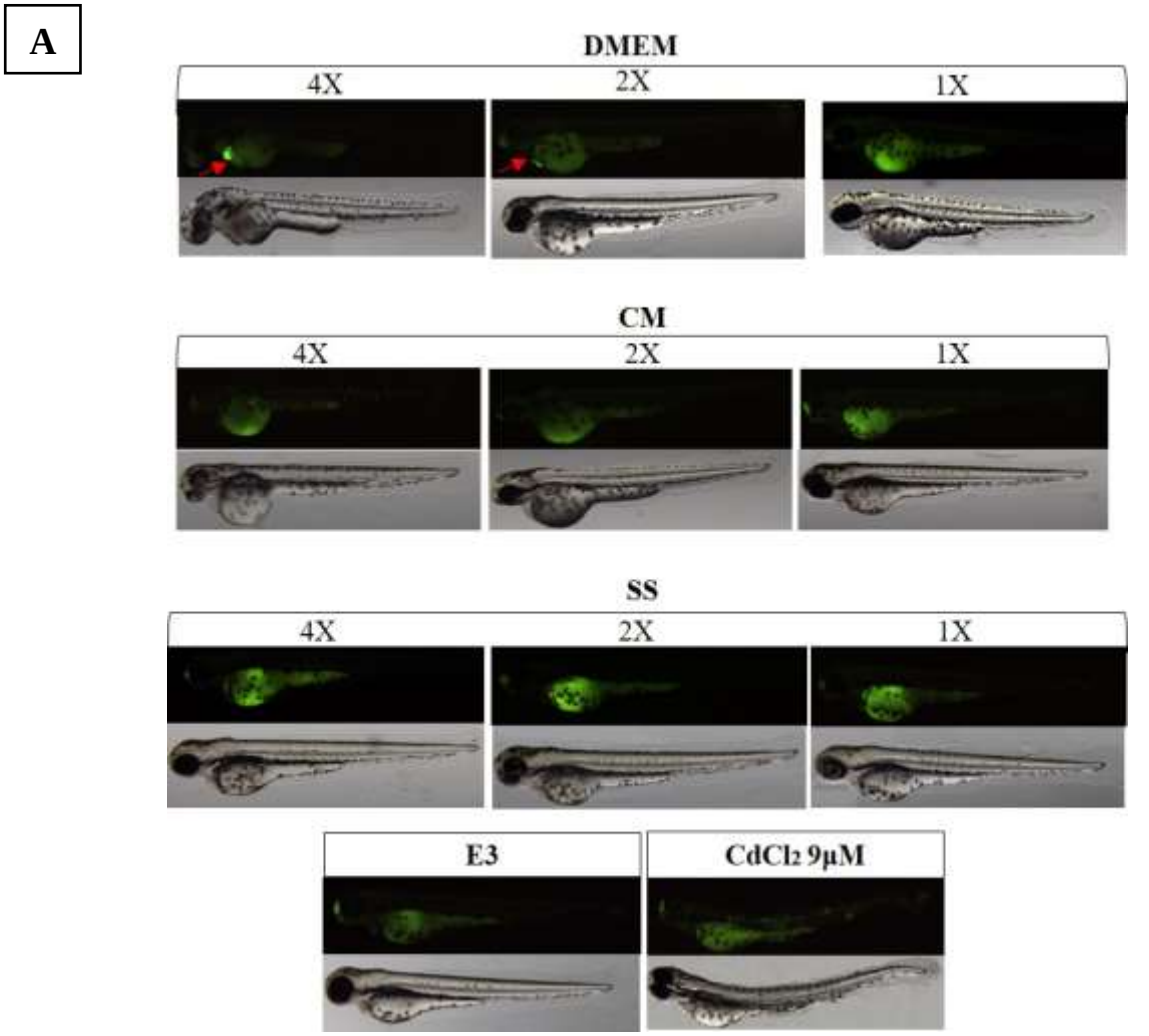

Figure 12. *Apoptotic assay by acridine orange vital staining.* ***(A)*** *Sample images of treated and control 72hpf-larvae. Red arrows mark the ectopic apoptosis induced by DMEM exposure.* ***(B)*** *Details of the images at higher magnification. Red arrows indicate the apoptotic spots.*

Alive 120hpf-larvae with minor o none visible morphological defects were selected from each experimental group for behavioural analysis. As reported in **Fig. 13**, we registered a maximum average reduction of 0.2 mm/sec in the locomotor activity of DMEM-treated embryos compared to the controls. However, based on preliminary tests and literature (Chen et al., 2011; Félix et al., 2018; Capriello et al., 2019) this is likely an irrelevant variation. Taken together, these data indicate that neither DMEM nor CM exposures produce neurotoxic effects.

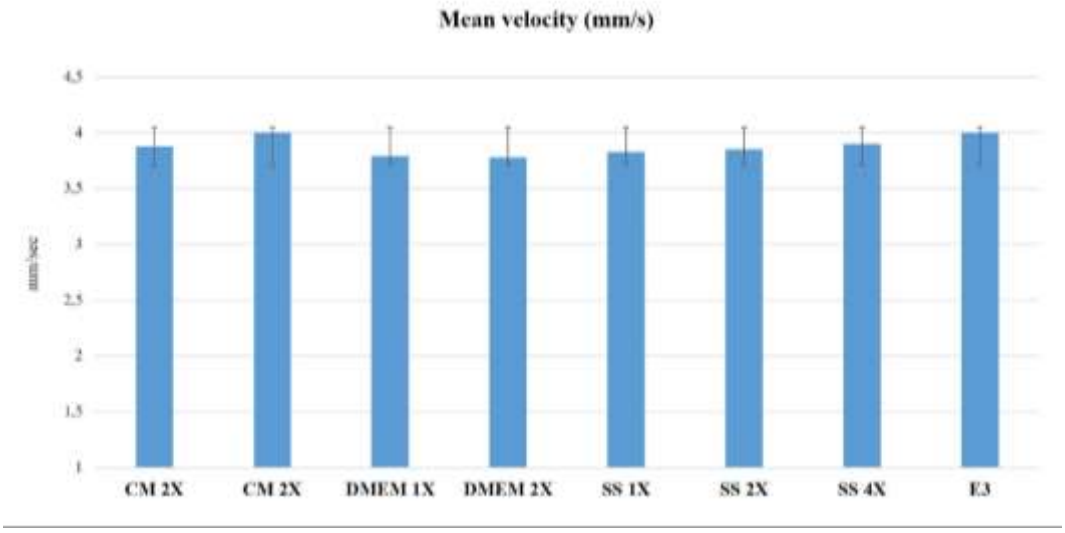

Figure 13. *Histogram showing the mean velocity of the larvae during a 15 minutes assay. Data are reported as the mean 10 120hpf-larvae per experiment. Raw data were processed with ViewPoint® FastData Manager (version 2.4.0.2510) and the charts drawn with Microsoft Excel 2016. Error bar = ± SD.*

Because of the lethal exert inflicted by treatment with DMEM at the highest concentration, we focused on the 2X experimental condition (including exposure to DMEM, CM and saline solution, as well as unperturbed controls) for downstream molecular characterization. In particular, we paid attention to some selected genes known to be involved in biological processes required for normal embryo development, such as apoptosis, neural development, inflammation and response to oxidative stress (**Tab. 2**). We also selected distinct reference genes to be used as internal control for the RT-PCR assay.

Biological process	Name	Abbreviation	Bibliography
APOPTOSIS	B-cell lymphoma 2 apoptosis regulator a	*bcl2a*	Lu et al., 2011 Miccoli et al., 2015
	Tumoral protein 53	*tp53*	Berghmans et al, 2005 Espín et al., 2013
	Tumor Necrosis Factor Receptor Superfamily Member 1A	*tnfrsf1a*	Espín et al., 2013
NEURODEVELOPMENT	Brain-derived neurotrophic factor	*bdnf*	De Felice et al., 2014
INFLAMMATORY RESPONSE	Interleukin-8	*il8*	Cortes et al., 2016 Basu et al., 2020
RESPONSE TO OXIDATIVE STRESS	Catalase	*cat*	Krysko et al., 2010 Wang et al., 2011
REFERENCE GENES	18S rRNA	*18S*	Tang et al., 2007
	Beta Actin	*β-act*	
	Ribosomal protein L13	*rpl13*	

***Table 2**. List of the genes considered in our experiments. The table indicates the main biological process involving each gene.*

Exons and promoter sequences of each selected gene were identified by BLAST searches on the Ensembl (http://www.ensembl.org/Danio_rerio) public annotated sequence database. For the RT-PCR assay, primer pairs targeted for Expression Sequence Tags (ESTs) were designed in the proximity of exon boundaries to avoid any non-specific amplification due to potential genomic DNA contamination. For the ChIP assay, primer pairs were picked on the basal promoter region of each gene, upstream of the Transcription Start Site (**Fig. 14**). The sequences of all primers were further checked using Primer Blast (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) to ensure the absence of non-specific amplicons.

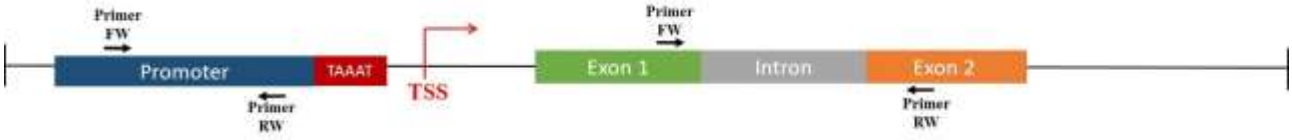

Figure 14. *Schematic drawing highlighting gene regions used for primer design. Primer FW= Primer Forward; Primer RW= Primer Reverse; TAAAT= TATA box; TSS = Transcription Start Site.*

For both molecular analysis, we used 30hpf-embryos because, at this developmental stage, the somitogenesis is complete, major vital organs are mostly formed (Kimmel et al., 1995), and the principal epigenetic patterns have been established (Balasubramanian et al., 2019).

Fig. 15 shows the mRNA abundance of the target genes in samples derived from embryos exposed to 2X concentration of DMEM, CM and saline solution, as well as from control unperturbed embryos. Consistent with the behavioural data showing the absence of neurotoxic effects induced by CM and DMEM, *bdnf* mRNA abundance remained constant in all the experimental groups. Inflammatory response was probably not stimulated under the described experimental conditions, since no variations were found in the expression level of *il8*.

Unexpectedly, a similar outcome was obtained for *bcl2*, *tp53* and *tnfrsf1a*, suggesting that apoptotic pathways involving these genes were not affected. The apparent discordance between this result and evidence obtained from the apoptotic assay could be ascribed to the distinct developmental stages considered for these two analysis. It could be argued that, probably, DMEM exposure triggers ectopic apoptosis in a developmental time window between 30 and 72hpf.

Intriguingly, the exposure to CM specifically increased the mRNA abundance of *cat*, suggesting a role for CM in the modulation of oxidative stress response.

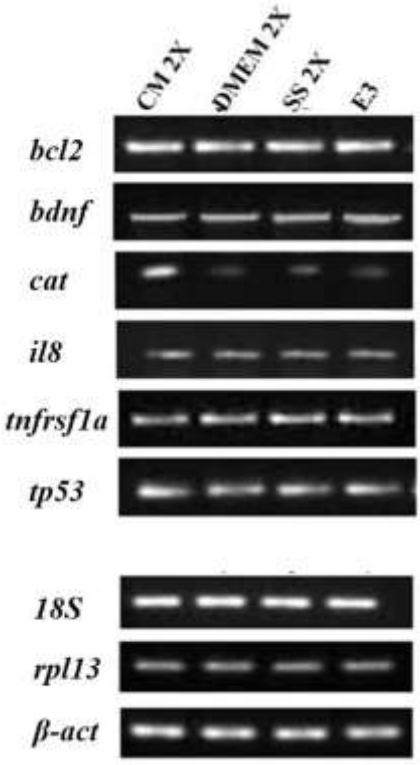

Figure 15. *Semi-quantitative RT-PCR showing the expression level of the analysed genes transcripts in 30 hpf-embryos at the indicated treatments. 18S, β-act and rpl13 were used to control gene expression.*

These findings were further supported by ChIP analysis aimed to the investigation of epigenetic marks associated to transcriptional activation and maintenance of euchromatin, such as acetylation of H4 (H4ac) and trimethylation of H3 lysine 9 (H3K4me3), and the negative epigenetic mark trimethylation of H3 lysine 9 (H3K9me3), associated with heterochromatin.

In particular, ChIP assays highlighted unchanged accumulation of the mentioned epigenetic marks at the promoter of *bcl2*, *tp53* and *tnfrsf1a* in chromatin samples derived from all the experimental groups, confirming the unaffected transcriptional outcome for these genes (**Fig. 16**). Most importantly, these experiments also revealed a concordant and specific increase in the occupancy of both H3K4me3 and H4ac modified nucleosomes at the promoter of the *cat* gene in CM 2X-treated embryos. In strict accordance, the negative epigenetic mark H3K9me3 was almost depleted in nucleosome occupying the same promoter region. Altogether, these findings well justify the increased amount of *cat* transcript specifically detected following CM exposure.

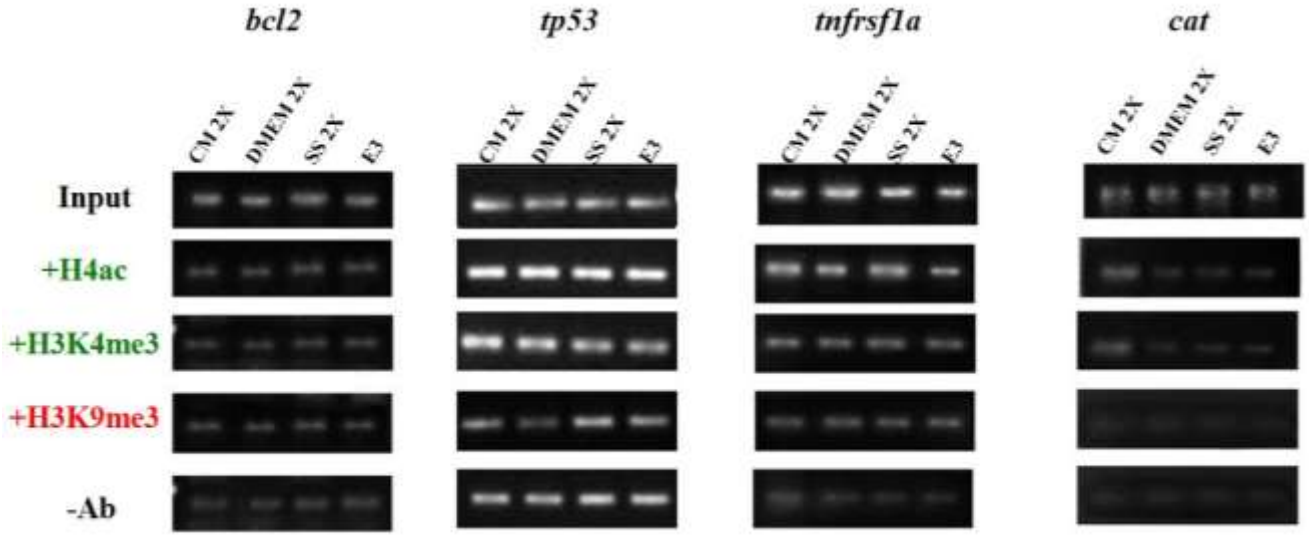

Figure 16. *ChIP-PCR analysis of promoter occupancy by H4ac, H3K4me3 and H3K9me3 for the indicated genes.*

CONCLUSIONS

To date, the zebrafish model is the main available high-throughput vertebrate assessment system with high translational power on humans. Moreover, the transparency of the embryos and their rapid development are uniquely suited for real-time *in vivo* studies of toxicity (Garcia et al., 2016; Cassar et al., 2020). For these reasons, the design and implementation of screening formats on zebrafish, targeting a wide range of pathways and endpoints (e.g. teratogenicity, cardiotoxicity, neurotoxicity etc.), has grown steadily in recent years. A continuously increasing trend in scientific publications in the field of zebrafish toxicity is also observed, with latest yearly reports being four times those from ten years ago (Cassar et al, 2020).

In this scenario, we took advantage of the innovative equipment available in the Zebrafish Laboratory recently established at the University of Palermo to devise a multi-parametric assay platform for the *in vivo* toxicological analysis of potential therapeutic compounds and new drug candidates. This experimental workflow combines different approaches to achieve rapid and simultaneous analysis of morphological, apoptotic, behavioural and molecular changes inflicted by multiple compounds at different concentrations during zebrafish embryogenesis. The robustness of this pipeline has been validated using distinct water-soluble chemicals (including cadmium chloride, dimethylsulfoxide, ethanol and tricaine) known to induce developmental aberrations in zebrafish at specific concentration ranges (Chen et al., 2011; Monaco et al., 2017; Ramlan et al., 2017; Félix et al., 2018; Capriello et al., 2019).

This successful validation step encouraged the application of our assay to evaluate comprehensively the developmental effects of CM derived from WJ-MSCs. The importance of such a complex biological mixture deals with recent characterization on mammals, highlighting that the use of CM offers a therapeutic alternative to direct stem cells transplantation as it provides broadly similar effects (Vizoso et al., 2017; Chudickova et al., 2019; Hsieh et al., 2013; Stefańska et al., 2020).

Comparative analysis of zebrafish embryos exposed to CM and DMEM (which is the non-conditioned counterpart medium), comprehensively suggests favourable effects of CM during development. These beneficial effects are fully justified considering that the MSC-derived CM generally contains trophic factors, chemokines, anti-inflammatory cytokines, anti-apoptotic factors, etc. (Kupcova, 2013). Indeed, exposure to CM prevented the onset of ectopic apoptotic spots observed following treatment with the standard culture medium, and preserved both the expression of the neuro-specific marker *bdnf* and locomotor activity. These results seem to be generally in line with

similar data from the available literature. For example, exposure of adult zebrafish model of traumatic brain injury to CM derived from human umbilical cord perivascular cells elicited anti-apoptotic and neuroprotective effects, accelerating the recovery of the normal swimming activity (Liu et al., 2020). Another interesting finding derived from our investigations pertains the protective effect of CM against oxidative stress through the establishment of a permissive epigenetic environment on the *catalase* gene promoter, which allows specific and significant upregulation of *catalase* gene expression in CM-treated embryos. Accordingly, published evidence highlights that the effect of CM against oxidative stress in various experimental systems indeed occurs through the increased mRNA expression level of *catalase* and *superoxide dismutase*, which hydrolyze reactive oxygen species, thus attenuating cell damage caused by free radicals (Wang et al., 2011; Sohn et a., 2018; Li et al., 2019; Liu et al., 2020).

In sum, our findings provide a promising outlook for the therapeutic role of WJ-MSCs and encourage the employment of CM derived from WJ-MSCs for clinical use. On the other hand, several issues needs to be pursued. For example, the identification of the bioactive factors contained in the CM used in our experiments not only should greatly facilitate future understanding of the mechanisms influenced during embryogenesis, but it may also help explain the sporadic aberrations observed in the CM-treated embryos. Future work must also concern an extended gene expression analysis on further key markers, to better describe the beneficial molecular landscape determined by CM treatment. Finally, it would also be interesting to evaluate the ability of CM to rescue developmental injuries induced by exposure of zebrafish embryos to dangerous substances.

APPENDIX A

List of primers targeted for ESTs (Expressed Sequence Tags) and used for RT-PCR

Gene	Sequences (5'-3') Forward (F) and Reverse (R)	Length (bp)	Annealing Temperature (°C)	Fragment size (bp)
bcl-2	F: GATGGCGTCCCAGGTAGATA	20	62	167
	R: CGAGCACTTTTGTTAGGTATGA	22		
bdnf	F: TTGGCGAAGAGCGGACGAAT	20	62	154
	R: ATAGTAACGAACAGGATGGTCA	22		
cat	F: ATGAAGCCGAGAGAGAGCGT	20	62	154
	R: TCAGCGTTGTGTTTATCCAGG	21		
il-8	F: GATAACCTGCGGCGAGTGG	19	62	187
	R: AGAGTATCAATGTCTTCTACAGT	23		
tnfrsf1a	F: GCATCAGACATTGGCGGAAG	20	62	155
	R: CCCTAGAGGTTTGGATTCACA	21		
tp53	F: TAGGCTCAGGTTCCCGCAG	19	62	167
	R: TCTTATAGATGGCAGTGGCTC	21		
β-act	F: ATCACACCTTCTACAACGAGC	21	62	173
	R: GGCATACAGGGACAGCACAG	20		
rpl13	F: AGGTGTGAGGGTATCAACATC	21	62	170
	R: TTGGTTTTGTGTGGAAGCATAC	22		
18S	F: ATCAGATACCGTCGTAGTTCC	21	62	167
	R: TGGTGGTGCCCTTCCGTCA	19		

APPENDIX B

List of primers targeted for promotors and used for Chromatin Immunoprecipitation samples analysis.

Gene	Sequences (5'-3') Forward (F) and Reverse (R)	Length (bp)	Annealing Temperature (°C)	Fragment size (bp)
bcl-2	F: AGCATTATCCTTATGTCGGT	20	62	148
	R: GAACCAAGAAGTGTCGTAAC	20		
bdnf	F: TCAGTGGTCGTCAGGAGAAG	20	62	190
	R: AGTGAAGGCTCAGATGCTTATT	22		
cat	F: ATTCACAGCATTGGCAAAAGCA	22	62	111
	R: TGTAACAACTTGAGGCAACATTT	23		
il-8	F: CCTGTGGTCAGACAAACACG	20	62	184
	R: CGAAGACTGAACTGTGTGGTT	21		
tnfrsf1a	F: ACTGTTTGTAACTGTGAATAATCT	24	62	161
	R: GCGTTAAAGGCATTGTATTGTAT	23		
tp53	F: ACCGACTTTCAGTTTGTTCTGT	22	62	165
	R: CACTTGTTGACCTTGCGTTTC	21		
β-act	F: GTATTTCGTGAACACAAGAGGT	22	62	121
	R GCAGAGTTACAAGTGGCAGAA	21		
rpl13	F: AGCGACTGACGAATGTACCAT	21	62	184
	R: CTGTAGCAATATAGGTGAGCG	21		
18s	F: CGAATGTCTGCCCTATCAACT	21	62	118
	R: GGATGTGGTAGCCGTTTCTC	20		

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