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Optimization of drug metabolism in the metabolic zebrafish developmental toxicity assay (mZEDTA)

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Optimalisatie van geneesmiddelenomzetting in de metabole zebravis ontwikkelingstoxiciteitstest (mZEDTA)

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List of abbreviations

AMC	7-amido-4-methylcoumarin
CM-H_2DCFDA	Chloromethyl-2',7' –dichlorofluorescin diacetate
CYP	Cytochrome P450
DarT	Danio rerio Test
DCFDA	2',7' –dichlorofluorescin diacetate
DM	Dimethadione
dpf	Days post fertilisation
DTT	1,4-Dithiothreitol
EC_{50}	Effect concentration 50%
ECVAM	European Centre for the Validation of Alternative Methods
EDTA	Ethylenediaminetetraacetic acid
ELS	zebrafish Early Life Stage test
ER	Endoplasmic reticulum
EROD	Ethoxyresorufin-O-deethylase
EST	Embryonic Stem cell Test
FDA	Food and Drug Administration
FET	Fish Embryotoxicity Test
FETAX	Frog Embryo Teratogenicity Assay - Xenopus
GA	Gallic acid
GSH	Glutathione
GSSG	glutathione disulfide
HBSS	Hanks' balanced salt solution
HLM	Human liver microsomes
hpf	hours post fertilisation
ICH	International Conference on Harmonisation
LC_{50}	Lethal concentration 50%
MAS	Metabolic Activation System
MBT	Mid-blastula transition
mDarT	metabolic Danio rerio Test
MM	Micromass test
MOPS	3-(N-morpholino)propanesulfonic acid
MS-222	ethyl 3-aminobenzoate methanesulfonate
NOAEL	No observed adverse effect level
NRS	NADPH regenerating system
OECD	Organisation for Economic Cooperation and Development
OR	Odds ratio
PBPK	Physiologically-based pharmacokinetic model
PBS	Phosphate buffered saline
PI	Halt Protease Inhibitor single-use Cocktail

RA	Rosmarinic acid
RLM	Rat liver microsomes
ROS	Reactive oxygen species
RO-water	Reverse osmosis water
SAR	Structure-activity relationsip
Se-M	Selenium-methionine
SZ	Supersomes
TI	Teratogenic index
TM	Trimethadione
Tris-HCl	Tromethamine-HCl
WEC	Whole Embryo Culture assay
YSL	Yolk syncitial layer
ZEDTA	Zebrafish Embryo Developmental Toxicity Assay
ZFET	Zebrafish Embryotoxicity Test

Chapter 1: General introduction

1.1 Preface

Some drugs (pharmaceuticals) have the potential to cause birth defects and pharmaceutical companies are therefore obliged to assess the potential adverse effects of a drug candidate prior to exposing women of child-bearing potential. Until now, the regulatory requirement includes dosing of pregnant dams of a non-rodent and a rodent species to different doses of the drug candidate during the period of organogenesis and evaluation of their foetuses for malformations after C-section. This is very costly, time-consuming and not in line with the 3R principle. Therefore, regulators and pharmaceutical companies are currently investigating whether alternative methods could replace either partially or completely the *in vivo* assessments. The zebrafish embryo is considered to be a promising model. Zebrafish embryos/larvae are not considered laboratory animals up to the free-feeding stage[1] and development can be monitored *in toto* throughout the period of organogenesis. However, the model also shows some limitations in comparison to the mammalian *in vivo* studies, such as the lack of biotransformation capacity during a major part of organogenesis. As such, proteratogens, i.e. prodrugs that need bioactivation to acquire their teratogenic potential, could be missed in the zebrafish embryo and consequently provide a risk for pregnant women and their offspring. To overcome this drawback, an exogenous Metabolic Activation System (MAS) has been suggested for co-incubation with zebrafish embryos. However, the currently used metabolic activation systems prove to be toxic themselves for zebrafish embryos. Therefore, the potential causes of this embryotoxicity were investigated in this thesis in order to develop a MAS that could be used by pharmaceutical companies as an add-on for zebrafish embryos and as such improve the sensitivity of this alternative method. Focus of this project will be on pharmaceuticals and teratogenesis, but application of the zebrafish embryo assay can be expanded to other fields of research, which will be elaborated upon in the general discussion.

1.2 Developmental toxicity of pharmaceuticals

Teratogenicity (from the Greek word “teratos”, meaning monster) can be defined as malformations that occur in the developing embryo during the period of organogenesis. Nowadays, the term teratogenesis is less used and being replaced by developmental/embryofoetal toxicity. Malformations can be caused by several factors, such as genetic abnormalities, infectious diseases, radiation, drugs, chemicals, etc[2]. However, in most cases (65-70%) the cause is unknown. In this thesis, we focus on pharmaceuticals as teratogenic agents. A well-known example, and probably the greatest teratogenic tragedy involving drugs, took place around the 60s. In 1957, thalidomide was brought onto the market in West Germany[3]. This

immunomodulatory drug was mainly prescribed as a sedative or hypnotic. Sometime later, it became an over-the-counter drug in West Germany and during the 60s, it was sold in over 40 countries around the world[3]. Besides its sedative and hypnotic nature, thalidomide was effective in treating morning sickness in pregnant women. Quickly after, it became a popular drug against morning sickness, reports of women giving birth to babies suffering from phocomelia (limb defects) after use of thalidomide were increasingly recorded, with an estimated number of at least 10,000[4]. Phocomelia was observed as either stumps or no formation of the long bones at all. Other effects that were associated with thalidomide usage were eye malformations, deafness and heart defects amongst others[4]. Approximately 50% of the affected babies did not survive early childhood[5].

Due to this tragedy, regulatory agencies enforced stricter regulations concerning developmental toxicity testing of compounds. Before the thalidomide crisis, potential teratogenic effects were assessed by using only one single test species (predominantly mice or rats). After analysing the results of thalidomide in rats, no malformations were observed. This is due to the relative insensitivity of rats and mice to thalidomide[6]. After further studies in non-rodent species, the teratogenic potential of thalidomide became apparent. Therefore, the principles of developmental toxicity testing had to be elaborated and revised (as reviewed by Bailey *et al.*, 2005)[7]. In general, a pharmaceutical nowadays has to be tested in two species, one of which is mandatory to be a non-rodent species (ICH guideline S5)[8]. In most cases, rats and mice are the species of choice as a rodent species and as a non-rodent species the rabbit, which belongs to the *lagomorphs*, is often used[9,10]. Concerning sample size, a minimum of 16 litters should be evaluated (ICH guideline S5). Therefore, the sample size often consists of around 20 pregnant females per dose to compensate for e.g. possible stillbirths. This means that per compound, at least 160 pregnant dams are being used (*i.e*. 4 doses with 20 pregnant dams per dose, tested in two species). Concerning dose selection, a high dose that causes maternal toxicity, a low dose causing no discomfort for the mother and foetus (for the determination of the NOAEL) and one or more intermediate doses are selected[7]. Administration of the compound is carried out preferentially via the oral route, but occasionally other routes such as intravenous, subcutaneous or intramuscular administration can be used (ICH guideline S5). Near-term, the pregnant dam is euthanised, C-sectioned and the foeti are examined for external, visceral and skeletal abnormalities[11,12].

No single test species can be considered the ideal test species for extrapolation of teratogenicity data to humans[7,13]. This is due to the differences in metabolic patterns and placental transfer function, amongst others[13]. Some of the major anatomical and physiological differences are reviewed by Bailey and co-workers (2005)[7]. Also false positive and false negative results occur[7]. The general predictivity of *in* vivo

assays for known human teratogens approximates 60% whilst for known human non-teratogens it is 54%. However, since the rule of mandatory testing in at least one rodent and one non-rodent species was applied, not a single compound was marketed for human use without previous signals in these animal studies, which means that the two-species strategy has a high predictivity for human teratogenicity[14,15]. This is of great importance, as false positives and false negatives can have important consequences for pharmaceutical companies. Especially a false negative result should be avoided as this will put pregnant women and their offspring at risk. Other species (besides the five most commonly used animals: rat, mouse, rabbit, hamster and monkey) were attempted to be incorporated as possible test species, with little success[7]. Due to the large numbers of animals that are used in developmental toxicity testing of pharmaceuticals, alternatives are currently being explored, which will be discussed in the next section.

1.3 *In vitro* teratogenicity testing

Russel and Burch (1959), postulated the principle of the 3R's, which concerns the use of animal species and how we can improve the quality of life of laboratory animals and reduce the amount of animals used in science[16]. One R, *refinement*, covers the amount of distress and pain that laboratory animals suffer from. If no other option is available, animal experiments should be carried out with the smallest amount of distress and humane endpoints should be taken into account to avoid unnecessary suffering. *Reduction* concerns methods used to minimise the amount of animals used in a study, as *in silico* screening and power analyses before the onset of an experiment might avoid the use of an excess of animals. The third R, *replacement*, concerns alternative approaches to laboratory animal testing. If possible, one should always opt for a method in which no laboratory animals are subjected to experimentation.

For developmental toxicity testing, refinement should be considered for every experiment, but also in terms of reduction and replacement, considerable efforts have been undertaken. Computer based modelling combined with physico-chemical information of a drug can already give insight in a possible teratogenic potential or safety. Together with physiologically-based pharmacokinetic model (PBPK) and structure-activity relationship (SAR), testing strategies can be worked out and test species can be considered to some extent depending on the chemical class of the compound[7]. Another promising field, which keeps both replacement and reduction in mind, is *in vitro* testing. However, in the final concept paper of the ICH S5 R3 guideline, it is stated that *in vitro*, *ex vivo* and non-mammalian *in vivo* assays are not considered to be the default approach for developmental toxicity testing but might be considered for

regulatory purposes under limited circumstances[17]. Thus, efforts in the development of alternative approaches should continue.

Some of the main advantages of *in vitro* techniques are the low cost compared to *in vivo* testing, its high throughput potential and the small amount of test substance that is required[11–13]. In 1978, Wilson established the characteristics of an ideal *in vitro* screening system, which besides some minor modifications is still generally accepted these days (table 1)[18].

Table 1. Features of an ideal in vitro teratogenicity test system (adapted from Wilson, 1978)[18]

I	Simple and easy to perform, yielding interpretable results
II	Rapid, usage of large number of samples
III	Low incidence of false negative results
IV	Having relevance to mechanisms of teratogenicity
V	Involving some aspects of progressive development
VI	Usable with various types of agents
VII	Usage of intact organisms capable of absorbing, circulating and excreting chemicals

In this respect, there are three (embryo) culture assays that are being applied. First of all, the Embryonic Stem cell Test or EST, which makes use of (mouse) embryonic stem cells isolated out of early mouse embryos (typically the blastocyst stage)[19,20]. These self-renewing stem cells are pluripotent and have been shown to spontaneously differentiate in cells of all three germ layers in an *in vitro* setting, forming so-called embryoid bodies[19,20]. Because of the lack of spatially controlled signals, morphogenetic development is not occurring within these embryoid bodies. Hence, early developmental processes can be evaluated on a cellular level and give insight in the mutagenic, cytotoxic and embryotoxic potential of a compound[20]. Historically, three endpoints are assessed, being the inhibition of differentiation into functional cardiomyocytes, cytotoxic effects on 3T3 fibroblasts (derived from Swiss albino mouse embryos tissue) and the cytotoxic effects on stem cells[21]. There has been criticism about the extrapolation of the data as only a few tissue types are being examined, which is not representing an entire embryo. However, with altered medium formulations, it is now possible to direct the differentiation of the cells into different cell types such as neural cells, chondrocytes and osteoblasts[22]. Although validated by the European Centre for Validation of Alternative Models (ECVAM), the test is not yet mandatory, nor can it currently replace one of the animal models because of its limitations (e.g. no information can be gathered on the organismal

level, only on specific cell lines). However, the concordance of test results of the EST compared to *in vivo* testing can reach up to 85%[21].

A second *in vitro* assay for teratogenicity is the Micromass (MM) test, which makes use of limb bud cells and/or mid brain cells from chick, mouse or rat embryos[23]. Compounds are administered to these cell cultures in different concentrations and for varying times and both differentiation and cytotoxicity are examined. The rationale behind the test is that small foci of cells start differentiating in neurons or chondrocytes when they are cultured *in vitro* in a high density[24]. Therefore, normal cell behaviour, such as cell adhesion, division, differentiation, movement and so on can be analysed[23]. Besides direct exposure, this system allows indirect transplacental exposure, which can be considered to resemble better the *in vivo* screening for pharmacokinetics of a compound[25]. Another advantage is that this technique allows culturing of a small amount of embryonic tissue into homogeneously responding cultures in a low-volume setting (96-well plate)[26]. Depending on exposure length, compound selection and selection of the type of embryonic tissue, it has been reported that the sensitivity and specificity for the test varies between 60-90% for teratogens and 89-100% for non-teratogens[27–29]. The predictivity of the MM during the ECVAM validation study was 70%, which means the MM is the least reliable test compared to the other *in vitro* alternatives[30].

The most commonly used test is the WEC or Whole Embryo Culture Test (Figure 1). In the WEC, whole embryos are cultured at an early stage of organogenesis in a rotator-type bottle system (*i.e.* embryonic day 6.5-12.5 in the mouse or embryonic day 8.5-14.5 in the rat)[31]. A relative index of teratogenicity can then be calculated after three general endpoints are examined, which are mortality, malformation and growth inhibition[32]. Other endpoints such as mitotic inhibition, limited cell differentiation and changes in macromolecule synthesis amongst others can also be used but are less common as the correlation between these events and developmental toxicity is still under debate[13,32]. Nonetheless, the WEC has the highest concordance to *in vivo* testing among all three alternatives, with a concordance of more than 90%[21,33,34]. Moreover, even though pregnant dams are still required, the reduction in animals used in the WEC compared to *in* vivo testing is approx. 60%[33]. Thus, WEC assay certainly has its value as a part of a test battery (whether or not in a tiered approach) of teratogenic tests but is unreliable as a sole predictor for teratogenicity as, e.g. examiner bias and selection of dosing regimen may influence scientific outcome and even though throughput is slightly higher compared to *in vivo* studies, the technique is still laborious and extrapolation to the *in vivo* situation is not straightforward[13].

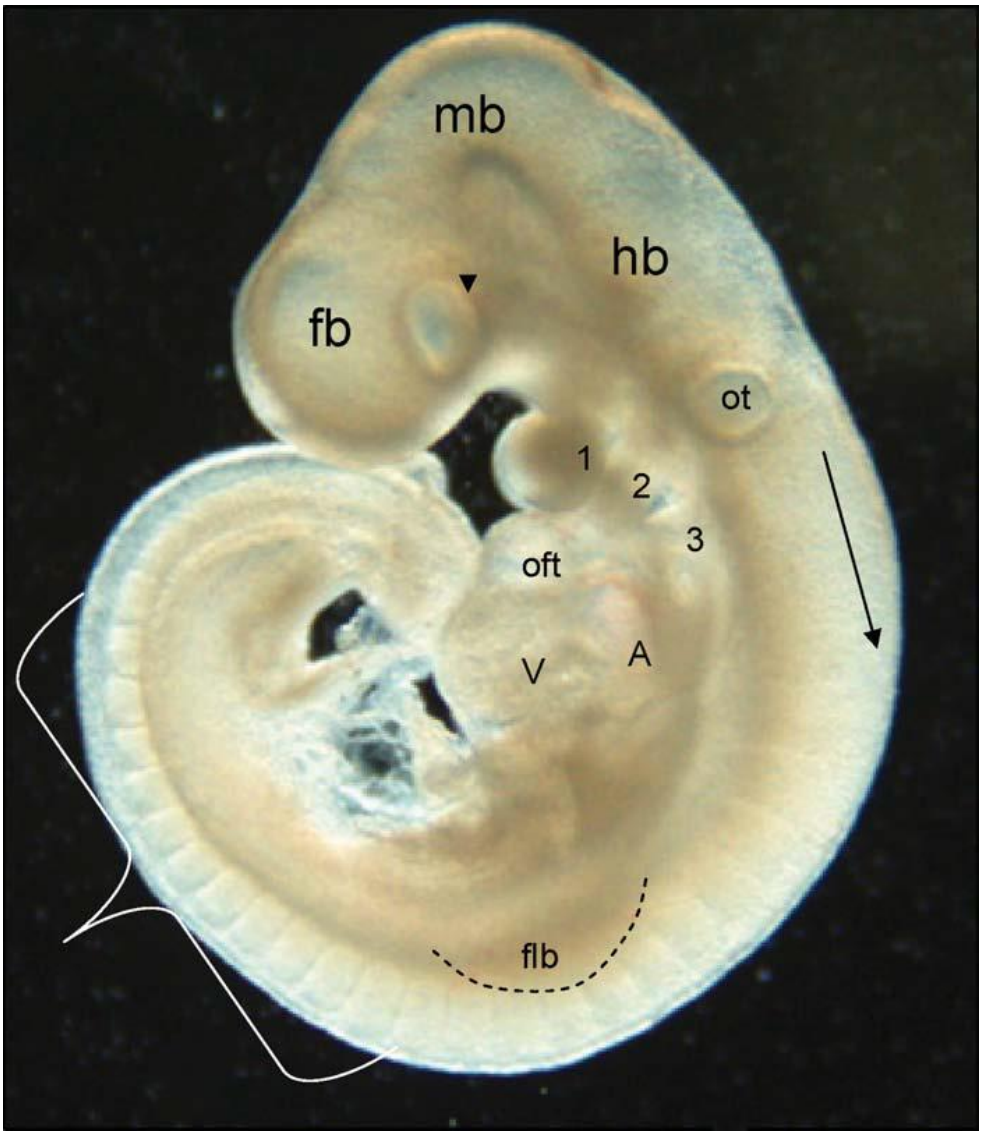

Figure 1. Whole embryo culture of a murine embryo (20-25 somite stage). At this stage, distinct structures can be evaluated. 1-3: Pharyngeal arches, A: atrium, fb: forebrain, flb: fore limb buds, hb: hindbrain, mb: midbrain, oft: outflow tract of the heart, ot: otic placodes, arrow: primitive spinal cord, arrow head, optic placode, brackets: pairs of somites. Copied from Augustine-Rauch *et al*., 2010[33]

In conclusion, *in vitro* testing of developmental toxicity certainly found its place in drug development, with the low cost, higher throughput and lower use of laboratory animals but care should be taken, as they do not yield information on pharmacokinetics, nor do they mimic the *in vivo* situation (e.g. role of maternal metabolism). Ideally, these tests can be used in a tiered screening strategy or as an add-on for specific compounds. When comparing all three *in vitro* tests from a pharmaceutical point of view, the WEC was preferred over EST, with MM being the least reliable test[13]. This is because a false negative classification is, in terms of the regulatory and industry perspective, the most critical. The rate of false positive results

is less important but should ideally be low. Thus, when looking at the rate of false negative and -positive results, WEC proves to be the best out of all three *in vitro* tests[21,30].

Around the year 2000, two different whole embryo tests have gained interest from the pharmaceutical industry. One of them was the FETAX (Frog Embryo Teratogenesis Assay - Xenopus), the other one is called the zebrafish embryo assay. They are somewhat comparable to the WEC, as an entire embryo is cultured outside the dam [30,32,35]. Both of these species have an external fertilisation and development of the eggs. Therefore, these tests are sometimes called 'alternative *in vivo*', as the early life stages of these animals are not considered laboratory animals and tests can be carried out on the whole embryo[36].

The FETAX makes use of the South African clawed frog, *Xenopus laevis*, as an amphibious laboratory animal model[37,38]. The duration of the test is as short as 96 hours and covers the entire period of organogenesis (Figure 2)[32,39]. A major advantage over the rat WEC is that large numbers of offspring can be produced by a single female. Moreover, the required amounts of compound are lower compared to *in vivo* testing as the test can be carried out in well plates and morphological examination of the early life stages is simple as the eggshells are transparent. FETAX is mainly used for environmental risk assessment[37,40]. Especially complex mixtures, found at waste sites can be analysed by this model with a high predictivity. However, it was also aimed to be used by pharmaceutical companies to study teratogenicity as well.

Figure 2. Different stages of the embryonic development of Xenopus in the FETAX. (Copied from: https://ntp.niehs.nih.gov/pubhealth/evalatm/test-method-evaluations/dev-tox/fetax/brd/index.html)

A three-phase validation of the FETAX has been carried out with one of the main conclusions being that the FETAX is a good alternative model for developmental toxicity testing, but that observer training is very important in order to reduce observer bias on morphological alterations[37]. A disadvantage of the FETAX is the fact that the test is not able to properly detect digital or limb effects. Only skeletal kinking might hint towards limb defects in mammals, but is not a guarantee[41]. Another disadvantage is the fact that this amphibian model is evolutionary quite remote from mammals and extrapolation of the results might, thus, be more complex. The most important disadvantage is probably the lack of a maternal metabolising component. As development is external, and therefore exposure is direct, no effect of maternal metabolism can be examined and pharmacokinetics can be completely different (e.g. no first pass effect)[32]. A possible solution to this in the form of an exogenous metabolism activating system (MAS) has been extensively tested and will be addressed later in this introduction. Nonetheless, the FETAX is not commonly applied any longer. This is due to some disadvantages such as the partial transparency, making it difficult to evaluate certain organ systems, the increased sensitivity of the later developmental stages compared to other aquatic organisms, excessive variability among laboratories concerning LC_{50}, EC_{50} and TI values and the fact that the toxic response is sometimes completely different compared to the response present in mammals[42].

The zebrafish embryo assay is a test that is being increasingly explored because of the advantages accompanied by *in vitro* testing[43]. Just as in the FETAX, embryos and larvae can be directly exposed to a compound in a small volume (48-well or even 96-well plate). The zebrafish embryo assay was first introduced in the late '80s, where the test was formerly called the 'zebrafish early life stage test' or ELS[44]. Its main purpose was to study (developmental) toxicity of environmental pollutants, but from the '90s onward, zebrafish found their way into teratogenicity screening and around the year 2000, they were introduced in pharmaceutical companies as well. Nomenclature can be quite deceiving, as the test introduced by Nagel in 2002 was called the Danio rerio Test (DarT)[45] and was intended for acute toxicity screening. In this assay, zebrafish are exposed from 2 hours post fertilisation onwards. On the other hand, the Zebrafish Developmental Toxicity Assay (ZEDTA) was used to denominate the zebrafish teratogenicity assay[46,47] in which exposure is only carried out from the onset of organogenesis (*i.e.* $5^{1/4}$ hours post fertilisation). Moreover, a third term was introduced for a zebrafish assay with a metabolic component, namely the metabolic Danio rerio Test or mDarT, in which exposure of a test substance to zebrafish embryos is also carried out from 2 hours post fertilisation onwards[48]. General predictivity of teratogenicity of the DarT is good, with a concordance that can reach up to 85% which makes it comparable to the EST and approximating the WEC[21,46].

Even though the zebrafish has some distinct advantages over other test species and alternative methods, the model is not perfect. Exposure and more specifically internal concentration of the parent compound and its metabolites remains a matter of debate. In mammalian studies, maternal concentrations of test compounds can be determined in the blood and for most small-molecule pharmaceuticals, exposure of the foeti is comparable to the C_{max} levels in the maternal blood. However, in the zebrafish embryo assay, exposure is carried out via supplementation of the compound to the medium and is dependent on the solubility of the compound, its potential to pass the chorion as a barrier and the potential to enter the developing zebrafish embryo (e.g. via passive diffusion). This means that using the mammalian C_{max} concentration in this assay does not necessarily mean that the embryos are exposed to the same extent as mammals. Another concern is the lack of endpoints compared to mammalian testing. Whereas assessment in mammals is carried out for external, visceral and skeletal malformations, assessment in zebrafish remains rather limited. Morphological assessment includes several endpoints, but one of the major shortcomings is the lack of skeletal assessment. This has recently been addressed by a task force of the European Teratology Society in which experts in the field of developmental toxicity are exploring the added value of skeletal examination in the zebrafish embryo assay (paper in preparation). It is known that some compounds give rise to false-negative results in zebrafish compared to *in vivo* mammalian data and that *in vivo* effects mainly consisted of by skeletal malformations. Another disadvantage, which is also the case for the WEC, is the lack of a maternal component. Moreover, the capacity of zebrafish embryos to metabolise compounds remains a matter of debate as there are reports claiming zebrafish are capable of metabolising substances, as well as zebrafish being incapable of metabolising compounds to a relevant extent. Metabolism and biotransformation will be discussed later in this introduction.

Even though shortcomings still exist in the zebrafish embryo assay, efforts are ongoing on improving its applicability in pharmaceutical research, amongst others. The results of these efforts will hopefully lead to an optimised test that has its value in the scientific field.

1.4 The zebrafish

1.4.1 General aspects

The zebrafish (*Danio rerio*) is a small (± 4 cm) freshwater fish belonging to the family of the *Cyprinidae* (or ray finned fish), part of the *Osteichthyes* (bony fish or teleosts) superclass. They are indigenous to small calm streams, ponds and even rice fields in the Southern parts of Asia, including India, Nepal, Bangladesh, Pakistan and Myanmar[49,50]. The first report on zebrafish was made by Hamilton in 1822, who described the newly discovered species during studies of the Ganges river delta[51]. Zebrafish can be distinguished by their blue horizontal stripes (Figure 3). The appearance of male fish is in general more torpedo-like, with the fins often having a yellow shade; whilst females have a more rounded belly and white fins[51].

Figure 3. Male (left) zebrafish can most easily be distinguished from female (right) zebrafish by their torpedo-like appearance. (Pictures provided by Zebrafishlab, University of Antwerp)

In the wild, zebrafish breed during the monsoon season, during which excessive rainfall causes the conductivity of the rivers to decrease. This, together with the sun rising in the morning is a strong trigger for females to spawn eggs. However, females will only spawn eggs in the presence of males, as pheromones into the water stimulate ovulation[52]. While kept in captivity, the fish are not dependent of the monsoon season anymore as they are maintained in ideal water conditions throughout the year. Therefore, females are capable of producing eggs all year round, laying up to a couple of hundred of eggs every week. For a review on zebrafish husbandry, see Lawrence 2011[53].

For research purposes, zebrafish are an interesting species. The last three decades, they are being used in a variety of scientific fields such as ecotoxicology, genetics and behavioural studies[54–56]. This is mainly because this vertebrate model has some distinct advantages over other laboratory animals. Zebrafish are cheap and rather easy to maintain for research purposes[53]. Also, the amount of test solution needed when they are applied in developmental studies is much smaller because of the small size of the fish[57]. This is particularly important in early drug development. In 2001, sequencing of the entire genome of the zebrafish was initiated at the Welcome Trust Sanger Institute. In 2013, a reference genome for the zebrafish was published, with a percentage of zebrafish orthologues compared to humans of 70%[58].

Therefore, the zebrafish gained interest as a model for genetical studies. Moreover, zebrafish are well suited for morphological assessment of early vertebrate development. Not only the chorion surrounding the zebrafish embryos is transparent, but during the earliest life stages the embryos and larvae are transparent as well. This allows easy longitudinal morphological examination of vertebrate development. Moreover, organogenesis is almost entirely completed after 96 hpf (hours post fertilisation). As zebrafish embryos are the main research topic of this thesis and morphological scoring of the embryos represents an important part of the work carried out in the lab, a thorough understanding of the development of zebrafish embryos and larvae is crucial. Therefore, zebrafish development will be discussed in the next paragraph.

1.4.2 Zebrafish development

The transparent zebrafish eggs are fertilised externally by the males who shed their sperm in the water while swimming in close proximity of a spawning female. The eggs will drop to the bottom of the tank. In captivity, zebrafish will eat their own eggs. Therefore, it is important to the adult zebrafish in a net or a special breeding tank so that they cannot access the eggs that are on the bottom of the tank. The embryo will start growing with the first cell cycle completed within 40 minutes after fertilisation[59]. Between 48 and 72 hpf (hours post fertilisation), the embryos hatch and reach the larval stage. Around 120 hpf, the complete digestive tract is formed and both the mouth and anus are opened. Moreover, the energy storage of the yolk is almost entirely consumed by that time after which the fish have to start preying small organisms (e.g. paramecia)[59]. The posterior chamber of the swim bladder should by this time be inflated and around 21 dpf (days post fertilisation), the anterior chamber inflates as well[60]. The juvenile fish reach sexual maturity after 5 months. The average lifespan of a zebrafish in the wild is slightly shorter because they are preyed upon, but in captivity, zebrafish can easily grow two and a half years old (Figure 4)[61].

The morphological development has been extensively described by the group of Kimmel *et al.* (1995)[59] and is similar to higher vertebrates such as humans. Several stages can be described: zygote period (0-45 min), cleavage period (45 min-$2^{1/4}$ h), blastula period ($2^{1/4}$-$5^{1/4}$ h), gastrula period ($5^{1/4}$-10 h), segmentation period (10-24 h), pharyngula period (24-48 h), hatching period (48-72 h) and the larval period (from 72 hpf onward). The following paragraph will give an overview of some, but not all, events occurring during each developmental stage.

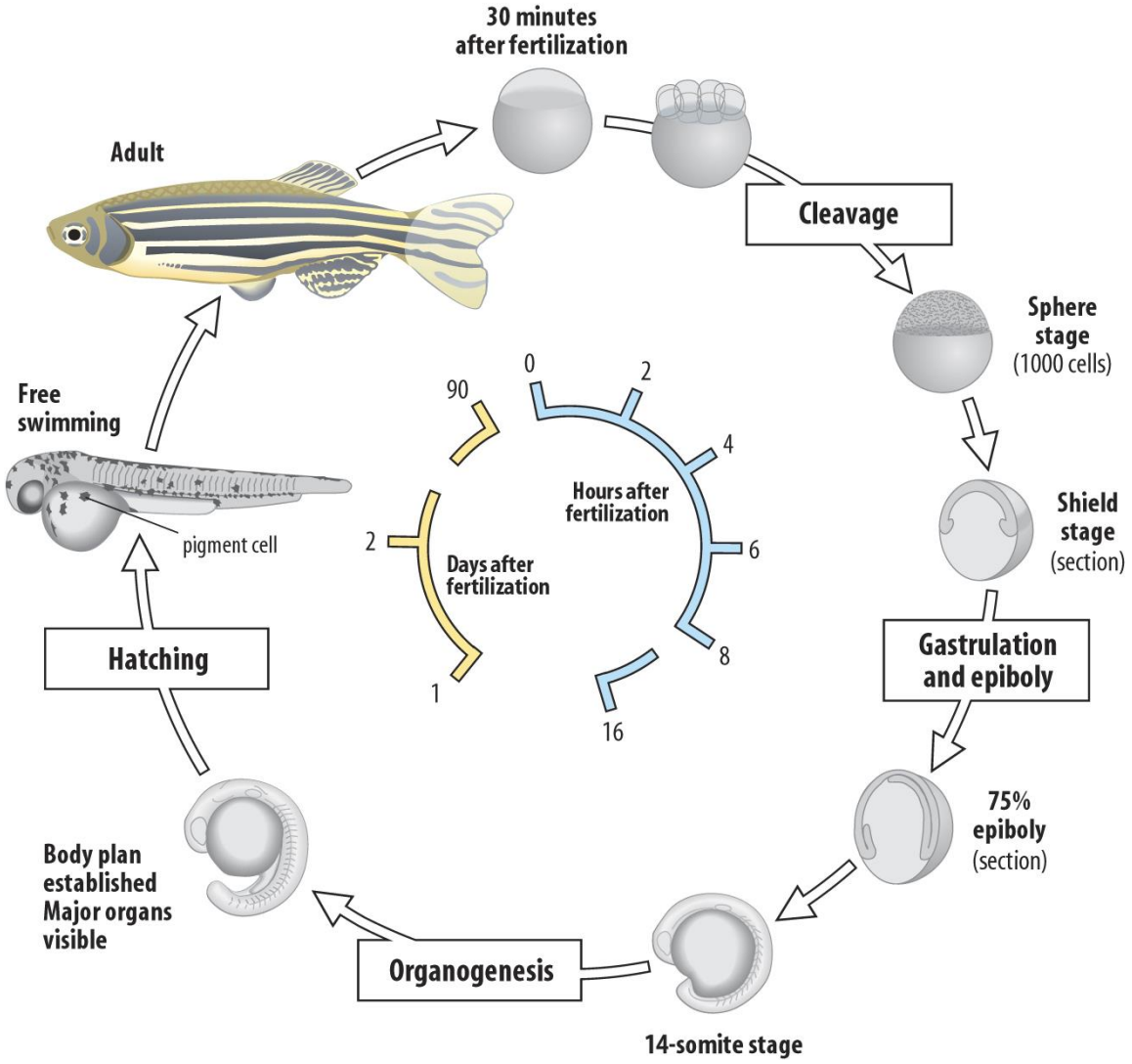

Figure 4. Zebrafish development.

Adapted from: http://www.mun.ca/biology/desmid/brian/BIOL3530/DEVO_03/ch03f09.jpg

The zygote period is when cytoplasm of the newly fertilized 1-cellular egg starts streaming towards the animal pole to form a blastodisc. After 40 minutes, the first cell division is completed and the embryo enters the cleavage period.

The cleavage period is defined by 6 cell synchronised divisions with formation of rather large, incompletely divided (meroblastic) blastomere cells that divide approximately every 15 minutes. Moreover, these cell divisions have a specific orientation (see Figure 5 & kimmel *et al.*, 1995)[59].

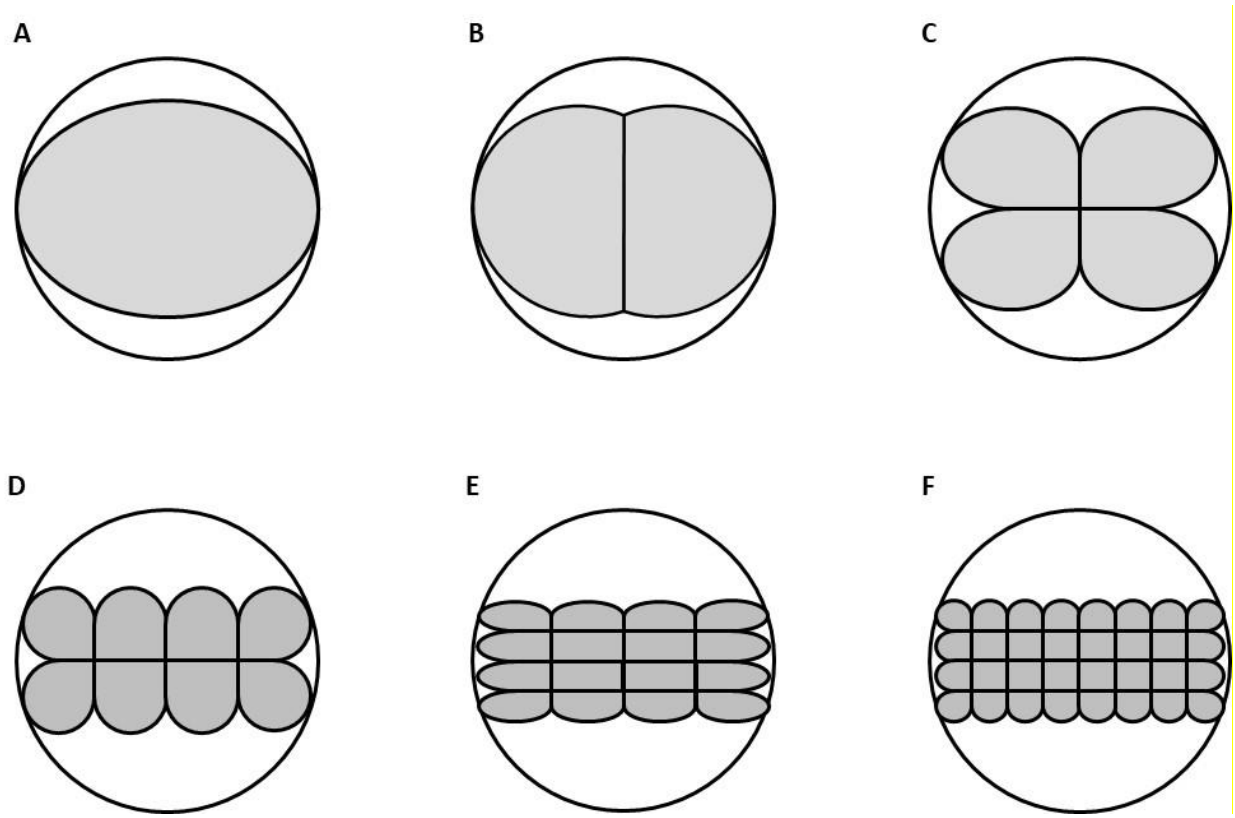

Figure 5. Dorsal view of the very first cell cycles during zebrafish embryonic development. **A**. One cell stage during the zygote period. **B-F** Representation of the first 5 cell cycles, which show a symmetrical division with an alternating cleavage axis along the short and long axis of the blastodisc. The dividing cells of the blastodisc are represented in light grey. The yolk is transparent. Adapted from Kimmel *et al*. (1995)[59].

The cleavage period is followed by the blastula period in which the blastomeres continue to undergo cell division, though the blastomeres can from now on divide asynchronously (which marks the onset of the mid blastula transition (MBT)). A structure that becomes apparent during the blastula period is the yolk syncytial layer (YSL), which is a feature unique to teleosts. It forms both an internal syncytium and external syncytium, with the latter being important for the start of epiboly. During epiboly, morphogenetic movements of cells occur, changing the blastodisc from a lump of cells to a cup-shaped multilayer of cells of uniform thickness from the animal pole towards the vegetal pole.

Somewhere around 50% epiboly, involution, convergence and extension (which are specific types of cell movement) occur, announcing the gastrula period. At this time, the three germ layers are being formed, as well as the embryonic axis. The germ ring surrounds the blastodisc and the embryonic shield situated at a specific position on this germ ring, can be observed. With the formation of the embryonic axis, germ

ring and embryonic shield, the fate map of the zebrafish can be established and one can tell which patch of cells will give rise to which organ/structure (figure 6).

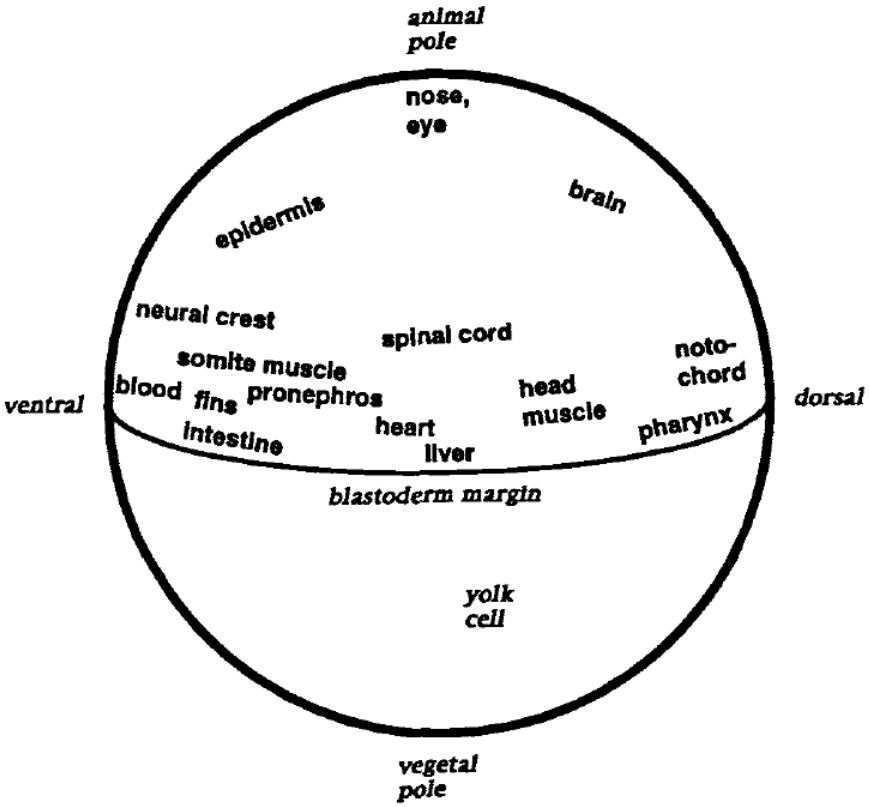

Figure 6. Fate map of the embryo during the gastrula period (Kimmel *et al*., 1995)[59].

Besides the obvious appearance of the tail bud, the gastrula period is also characterised by the formation of the first neural structures such as rudiments of both the brain and notochord[59].

After epiboly is completed, the embryo enters the segmentation period and the optic vesicles, pigmentation, fins and (rudiments of) all major organ systems start to develop. The tail bud is extending and the first body movements can be examined. The developing somites sequentially grow in the trunk and tail and the number of full-grown somites can be used for staging the embryo. The pace at which new somites are formed is around 2-3 somites/hour. At the end of the segmentation period, the majority of processes involved in neurulation are completed and a clear optic primordium, giving rise to the eye and an otic vesicle with the otoliths (for the poise of the embryo) can be examined on both lateral sides of the embryo[59].

The pharyngula period is the second day of life for the embryos and by now they have the classic vertebrate morphology. At this point, embryos from different species resemble one another the most. The notochord is, by this time, well-developed and the hollow nervous system is expanding anteriorly. Five brain lobes can be distinguished at the beginning of the pharyngula period. Besides the internal organs further developing, evidence of the fins can be examined. Sonic hedgehog genes, expressed in both fin buds from zebrafish and limb buds from mammals is the most significant connection between both. During the pharyngula period, the blood circulation further develops and blood cells can reach all the way into the tail tip. Heartbeat can be examined clearly and venous return to the heart can be observed by blood cells running over the yolk towards the developing heart. Zebrafish have a single tube folded into an S-shape that rhythmically contracts, propelling the blood cells unilaterally. The first pigmentation becomes apparent with the retinal epithelium being pigmented, as well as the melanophores who will also arrange in characteristic patterns that will give rise to the typical longitudinal body stripes of zebrafish. Where movement was still uncoordinated in the segmentation period, the embryo in this phase starts showing coordinated movements[59].

At 48-72 hpf or during the hatching period, embryos continue to develop further. However, hatching itself is not an accurate staging index as there is a rather fair amount of variation on this event, even between embryos of a single spawning event of a female. Controlled moving of the tail, together with enzymes from the hatching gland digesting the egg shell, enables the zebrafish to break and shed the egg shell, which is the transition from embryo to larva. Morphogenesis of the organ rudiments is now almost complete and, thus, slows down considerably (with the exception of the digestive system). The mouth is protruding and open at this time. However the anus is not yet opened and the digestive tract is still being formed[59].

During the early larval period, from the third day onward, the embryo continues to rapidly grow. Moreover, morphogenetic movement is mostly completed and the swim bladder should become visible and some days later should be inflated. This is necessary for the larva to control its location in the water and to help with swimming and hunting. Moreover, the entire energy reserve of the yolk is consumed and the larvae need to hunt for food. By that time, the digestive system is fully functional and the anus has opened. The zebrafish stomach, however, is more a bulbus along the digestive tract, mixing food with excretes, rather than a fully specialised structure as seen in mammals. The only organ system that, by this time, is still very immature is the reproductive system. Only after 60 dpf, the gonad differentiation is terminated[59,62].

The zebrafish embryo is already used by several pharmaceutical companies for screening of developmental toxicity[33,43,46,63–65]. The predictivity of the DarT is good. However there are some discrepancies in the testing strategies. Therefore, a consortium has been established with the goal to define a protocol and validate the inter- and intra-laboratory reproducibility. In general, most compounds were identically classified in all participating labs[65].However, the consortium did not look at the metabolism of the compound by zebrafish embryos, whilst this plays a pivotal role for teratogenicity screening. Namely, some compounds (proteratogens) need to be bioactivated in order to trigger any effects[66,67], while others can be quickly metabolised by the liver, reducing their teratogenic potential (teratogens)[68]. Therefore, we will address metabolism in the next section.

1.5 Metabolism and xenobiotic transformation

During *in vivo* mammalian teratogenicity experiments, embryos are indirectly exposed to the drug. The dose that reaches the embryo will be different from the dose administered, as it will be distributed over the dam and the foeti. Moreover, the compounds have to pass several maternal tissues that are capable of metabolising a significant part of a drug, such as the liver, intestine and placenta[69].

Metabolism of exogenous compounds is carried out in multiple phases. Phase 0 is considered to be the entry into the cell, whereas phase III is the export out of the cell and body. Metabolization is, thus, mainly carried out during phase I and II in order to facilitate excretion of the compound. The first phase is characterised by monoxidation reactions to increase water solubility of the product. In phase II, enzymes conjugate the molecule to make it even more water soluble[70]. The metabolites with an increased water solubility formed after both phases make that the compound can be more easily excreted by e.g. the kidneys[71]. Phase I metabolism is regarded as most important. Not only because it is the first step in detoxification, but it can also have the reverse effect, in which an oxygenated metabolite can become (more) reactive compared to its parent compound. In rare cases, it is only after phase II metabolism that a compound becomes reactive[72]. For pharmaceutical compounds, phase I-metabolism is mainly due to the cytochrome P450 (CYP) enzymes but also monoamine oxidases, flavin-containing monooxygenases and alcohol or aldehyde dehydrogenases[73].

The term cytochrome P450 was first coined in 1962 after discovering that a pigmented protein in a microsomal preparation, capable of binding carbon monoxide, showed a distinct spectrophotometric absorption at 450 nm[74]. This superfamily of endoplasmic reticulum-bound enzymes was found to have endogenous functions such as progesterone metabolism and metabolism of polyunsaturated fatty acids.

They are not only present in mammals, but in all animals, plants and fungi and even in some prokaryotes[74]. Due to the widespread nature of cytochromes and rapid discovery of different CYPs, a need for a systematic classification was met and in 1987, CYPs were named and divided into families and subfamilies based on their amino acid sequence[74,75]. The general length of the protein chain is around 400-500 amino acids and they are classified within the same family when more than 40% of the sequence is identical. Subfamilies have a protein similarity greater than 55%. The microsomal CYP enzymes are hemoproteins. At the amino-terminus, a hydrophobic 20-25 amino acid residue anchors the protein to the membrane. The function of CYPs is principally to monooxygenise various substrates. In order to do this, molecular oxygen, as well as a reducing agent is needed. The reducing agent is NADPH. The enzymatic mechanism of CYPs is cyclic and during oxygenation, two electrons are added to the cytochrome. The first one binds the oxidised P450 which has a substrate bound to reduce the iron atom of the heme, after which the second one binds to the oxygenated CYP to activate the oxygen bound to heme by splitting the O-O bond. This leads to one oxygen being bound to the substrate whilst the other is reduced to water (Figure 7). Even though CYPs can be found in cells throughout the body, they are most prominent in the liver and, to a lesser extent also in the kidneys, intestine and brain. In fish, they are present in the gills as well[75,76].

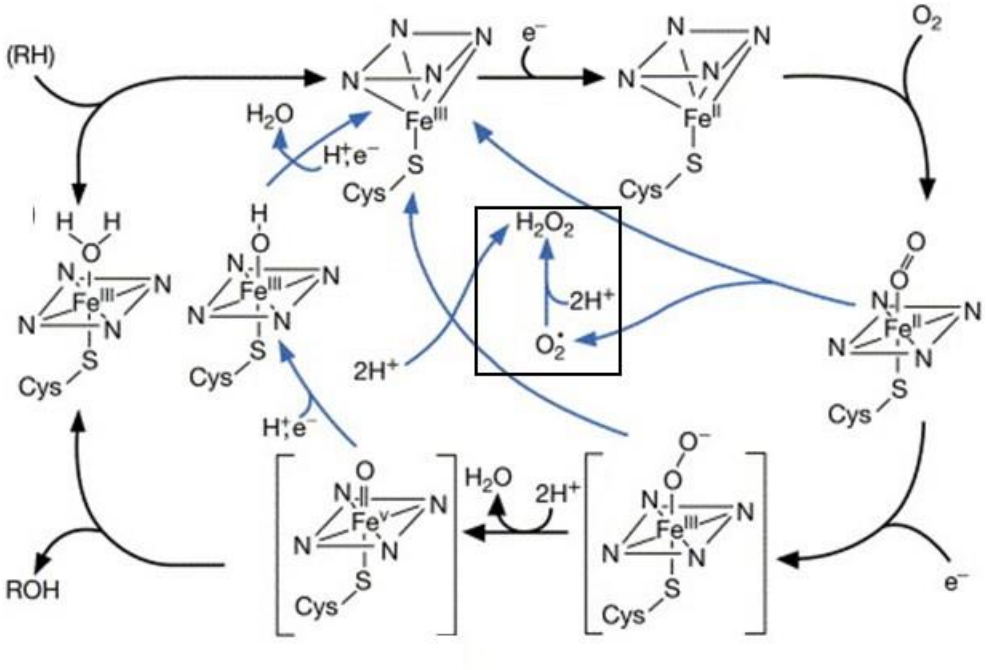

Figure 7. Cytocrome P450 cycle. The cycle is constituted of 6 steps in which oxygen and electrons are transferred from/to the heme group. Blue arrows show 'leaks' at which ROS can be produced. Square: production of hydrogen peroxide. Adapted from Hrycay and Bandiera (2015)[77].

In humans, 57 genes coding for CYPs have been identified of which 15 show metabolic activity towards xenobiotics[78]. The majority are derived from CYP families 1-3. However, there are 5 predominant CYPS that account for 95% of phase I drug metabolism (*i.e.* CYP1A2, 2C9, 2C19, 2D6 and 3A4)[78]. The most abundant one which also shows the highest diversity of substrates is CYP3A4. It can be induced via its PXR-receptor by compounds such as nifedipine and dexamethasone and besides drug metabolism, it has a key role in detoxification of hepatotoxins[79,80].

Currently, there are approximately 86 CYP genes identified in zebrafish, which can be divided over 17 families (Genome Reference Consortium (GRCz11))[81]. In terms of drug metabolism, families 1-3 are also in zebrafish the most important as they are responsible for the majority of xenobiotic transformation. A review of zebrafish CYPs, their function and synteny with humans and other species was published recently by our research group[82]. It became obvious that zebrafish were also capable of metabolising compounds and often, a direct ortholog of the human CYP could be identified. However, CYP3A65 which is the zebrafish counterpart of the most important human CYP (CYP3A4) is only 55% identical at the amino acid level[83]. Moreover, studies in our research group with midazolam, which is a CYP3A-specific marker/compound, showed that zebrafish show no metabolization of midazolam[84]. Therefore, there seems to be a distinct difference between human CYP3A4 and zebrafish CYP3A65. In terms of other CYPs, we proved that zebrafish are capable of metabolising compounds. The principal metabolite(s) formed were often the same as in humans, though their ratios might differ. For some compounds, completely different principal metabolites were identified but metabolization was present, nonetheless[84,85].

In terms of metabolism, there has been years of debate about the capabilities of zebrafish to biotransform compounds into their metabolites. CYP mRNA levels can be detected as early as 8 hpf[86]. However, mRNA levels may not be a good predictor for the actual concentration or activity of the metabolising proteins[84]. Recent results from our research group showed that concentrations of metabolites were either very low or negligible during organogenesis.

1.6 Exogenous metabolic activating systems (MAS)

In order to increase drug metabolism and potentially bioactivation in the zebrafish embryo assay, it has been proposed that an exogenous metabolic activation system (MAS) can be introduced for co-incubation with the zebrafish embryos. In this section, some commonly used options for MAS will be discussed.

A well-established method is the use of human hepatocytes. The first reports on hepatocytes being used for *in vitro* drug metabolism studies arose in the 70s with the isolation of rodent hepatocytes[8788]. Briefly, human hepatocytes were preferred over rodent hepatocytes but the availability of fresh tissue was limited and sources of tissue were mainly biopsy tissue from surgical interventions[89,90] and thus, studies were rather small and simplistic. It was only 20 years later that hepatocytes became very widely used, with the development of cryopreservation of human hepatocytes. Due to cryopreservation, a larger number of experiments could be performed with the same batch of liver tissue without the loss of enzymatic or transporter activity, which up until then was the major drawback[88]. This lead to acceptance by the FDA of this *in vitro* system for metabolism and drug-drug interaction studies[91].

Even though cryopreservation of hepatocytes was a major advancement, culturing hepatocytes without losing their cell-specific characteristics remains a challenge. After approximately 7-10 days, a significant loss of hepatic phenotype due to dedifferentiation occurs[88]. It was suggested that hepatocytes will dedifferentiate less fast when their natural environment is mimicked. Therefore, the classical monolayer of cells cultured in a plate was further elaborated upon and a first development was the sandwich cukture[92,93]. This culturing method enabled studies of biliary secretion of compounds. But the lifespan of the cells was not really prolonged. Very recently, the focus has been shifted to completely three dimensional cultures of cryopreserved hepatocytes that should mimic the *in vivo* situation to a greater extent. A 3D hepatocyte spheroid culture showed a prolonged differentiation state and lifetime of 28 days compared to the 7-10 days of monolayer culturing when perfused with culture medium[94]. Recently, a study published by Bell *et al.* (2016)[95] reported on a spheroid culture that retained hepatocyte characteristics for a total duration of 5 weeks. Beside preparation of the spheroids, the technique does not require any special needs, providing an excellent source for studying long-term drug exposure.

However, hepatocyte cultures still have some major drawbacks. The optimisation of the culturing system can be laborious, some required equipment can have a high price tag and human hepatocytes are currently only used for hepatotoxicity, drug metabolism and drug-drug-interaction studies and, to our knowledge, no reports on the use of human hepatocytes as a MAS for teratogenicity screening have been published[88].

An artificial cell fraction, the S9 fraction, can also be used as a metabolism activating system. The S9 fraction can be obtained from homogenates of cells, preferably hepatocytes, after centrifugation at 9000g, with the supernatant being the S9 fraction[96]. The pellet consists of lysosomes and mitochondria, whilst the nuclei and other large cell debris should already have been removed by a previous centrifugation step at 1000g. The ER and Golgi apparatus are still present in the supernatant meaning that both phase I and phase II enzymes are readily available in the S9 fraction. This includes the cytosolic enzymes such as aldehyde oxidase, xanthine oxidase and several transferases. In contrast to hepatocytes, cofactors such as β-nicotinamide adenosine dinucleotide phosphate (NADPH, phase I oxidation), uridine 5'-diphospho-α-D-glucuronic acid (phase II), 3'-phosphoadenosine-5'-phosphosulfate (phase II) and glutathione (GSH, phase II) should be added to the incubation mixture to achieve enzymatic activity. On the other hand, S9 fractions are cheaper, can be easily stored and throughput is greater compared to hepatocytes[96]. They also do not bring in the same complexity as a cell system, which can be both an advantage (easy access of the test compound to the enzymes without extra barriers such as the cell membrane) and a disadvantage (no monitoring of protein or mRNA-induction and no testing of compound uptake in the cell), depending on the aims of the study. Fluorogenic assays are available (e.g. 7-ethoxycoumarine-o-deethylase assay or ECOD) to quickly compare enzyme activities (specifically CYP activities) of different batches of S9 fraction. S9 fractions are mainly used in the Ames-test for mutagenicity[97,98] but are also applied for metabolic stability assays and have proven to be a reliable *in vitro* alternative to hepatocyte cultures[96]. However, they are not used in teratogenicity studies because of two reasons: 1. (pro-)teratogenicity is mainly (but not exclusively) dependent on CYP-mediated metabolism and phase II metabolism can be a confounder of the results. 2. There is a more purified cell fraction containing high concentrations of CYP enzymes that is readily available, namely (liver) microsomes.

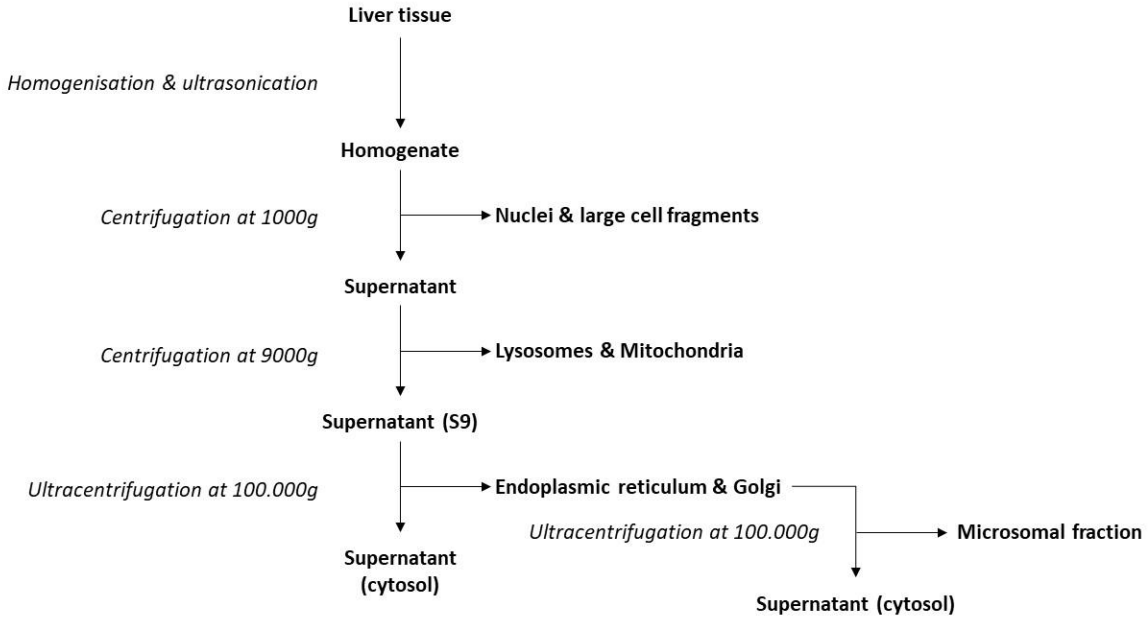

Figure 8. Steps in the preparation of specific liver subcellular fractions. Vertical arrows represent the supernatant, whilst horizontal arrows represent the pellet. Adapted from Richardson *et al*. (2016)[96].

Microsomes are, just like the S9 fraction, a cell derived artificial MAS. However, the purity of microsomes is even further increased as it consist mainly out of ER-membrane bound enzymes from which CYPs are the most abundant[96,99]. Microsomes can be obtained after further purification of the S9 fraction by two ultracentrifugation steps at 100,000g[39]. The pellet formed after the first ultracentrifugation is resuspended and ultracentrifuged a second time to have an extremely purified pellet that can be resuspended and stored at -80°C until further use (Figure 8). After resuspension, the microsomes are small vesicle-like structures which consists of membranes of the ER together with ribosomes and, as mentioned earlier, ER-membrane bound enzymes (such as CYPs) but also some flavin-containing monooxygenases which are indigenous to the ER[99,100]. Just like S9 fractions, microsomes are used in metabolism studies and metabolite profiling studies as well as in CYP inhibition studies[101]. Recently, some research groups started using the human liver microsomes (HLM) as a MAS for teratogenicity screening in both the FETAX and the DarT (called metabolic Danio rerio Test or mDarT when a MAS is added)[48,67,68]. The use of microsomes in the mDarT will be further discussed in the next section. One might at this point question why direct exposure of metabolites to the zebrafish embryos is not performed and thus, why there is a need for a metabolism activation system. Ideally, this would be the standard for pharmaceutical companies, however, but the approach is in practice not feasible. During early drug development the amount of compounds of interest is still large. Further on in development, only some will be retained for further

development. However, identification of all metabolites for a big pool of compounds is too labour intensive, as well as too expensive for a pharmaceutical company. Moreover, production of (some of) the metabolite(s) delays the process of drug development. Therefore, pharmaceutical companies will prefer a setup in which small volumes of compound are required and in which a human metabolite profile is generated without the immediate need for identification and production of these metabolites. Identification of the metabolites can then be carried out later in drug development for a smaller amount of products, when necessary.

1.7 Coincubation of zebrafish embryos with MAS

Since there has been and still is a lot of debate on whether or not zebrafish early life stages are capable of metabolising compounds, some research groups started testing a metabolic activation system (MAS)[48,67,68], *i.e.* liver microsomes. As indicated earlier, the need for a metabolic component is twofold. First of all, teratogens might be efficiently metabolised to their non-teratogenic metabolites during first-pass metabolism in the liver[102]. This means that if the zebrafish embryo assay lacks biotransformation, the embryos are exposed to the teratogenic parent compound and thus will develop teratogenic effects when this would not be the case in mammalian species, leading to false positive results. On the other hand and more importantly, if a compound is proteratogenic, it requires bioactivation (Figure 9).

Substance	Human phase I enzymes involved in substance metabolism
2-acetylaminofluorene	**CYP1**, 2A6
Benzo[a]pyrene	**CYP1A1, 1B1**
Aflatoxin B_1	CYP1A, 1B, 2A6, 2B6, **3A4**
Carbamazepine	CYP1A2, 2A6, 2B6, 2C8, 2C19, 2E1, **3A4**, 3A5, 3A7, 2C8, **2C9**
Phenytoin	CYP2C8, **2C9**
Trimethadione	CYP2C9, **2E1**, 3A4
Cyclophosphamide	CYP2A6, **2B6, 2C8/9**, 2C18/19, **2E1, 3A4**, 3A5
Ifosfamide	CYP2A6, **2B6, 2C8/9**, 2C18/19, **2E1, 3A4**, 3A5
Tegafur	CYP1A2, **2A6**, 2C8
Thio-TEPA	CYP2B6, **3A4**

Figure 9. List of several proteratogens and their respective enzymes responsible for phase I metabolization. Enzymes in bold are the principal metabolising enzymes for that compound. Adapted from Weigt *et al*. (2011)[35].

If no MAS is added and zebrafish are uncapable of biotransforming the compound, a false negative result might occur[68]. This can be catastrophic for a pharmaceutical company as it will invest great amounts of time and money into further development of the compound which later on turns out to be a teratogenic entity. Therefore, it is suggested that a MAS is implemented in the testing system and several research groups published papers on this topic[48,67,68].

However, there is one major drawback in all these studies, *i.e.* the exposure window. Ideally, *in vitro* testing with zebrafish embryos should mimic mammalian testing as closely as possible, meaning that the embryos should be exposed to the test compound during the entire period of organogenesis (thus, from $5^{1/4}$ hpf up until 96 hpf). All these research groups exposed embryos for shorter periods of time, after which the embryos are washed and then further developed in a non-toxic medium. The reason for the short exposure periods is because microsomal preparations are embryotoxic[48,67,68]. When zebrafish embryos are coincubated with liver microsomes without the addition of any test compound, they start to develop malformations and depending on the concentration of liver microsomes they can even coagulate[39]. Even though these articles agree on the toxicity of the MAS, the reason for the toxicity remains unclear. First of all, the coincubation temperature is 32°C, while 28.5°C is optimal for zebrafish embryonic development[59,103]. There is also evidence that a possible role of oxidative stress might be involved, as reactive oxygen species (ROS) are formed during the cytochrome P450 enzymatic activity (see square in Figure 5)[104]. In normal cell homeostasis, radicals are present and can even possess signalling properties[105]. However, there is a very strict balance between radical production and elimination, as an accumulation of radicals can have harmful effects. Increased levels of ROS, leading to oxidative stress, can affect cells on different levels. They can either have a direct effect on DNA, where an increased amount of ROS can cause DNA damage but also carbonylation of amino acids and proteins, and lipid peroxidation might occur[106]. This can be limited to cells, but increased ROS levels can also affect entire tissues and organ systems if the imbalance cannot be stabilised. As increased levels of ROS are a by-product of CYP activity and oxidative stress can have deleterious effects, it is not unlogic that zebrafish suffer from embryotoxicity as a consequence of elevated ROS levels during coincubation with MAS. This assumption will be one of the parts dealt with in this thesis.

In summary, in order to avoid false negative results in the ZEDTA, zebrafish embryos should be exposed to a drug and its metabolites during the entire period of organogenesis as no critical windows for a specific compound are missed. Therefore, it is crucial that the optimisation of the metabolic ZEDTA aims at a continuous coincubation system in a non-embryotoxic medium in which the embryos can be exposed to the compound.

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Chapter 2: Aims of the doctoral project

There is a growing demand for alternative assays in order to reduce laboratory animals used in *in* vivo developmental toxicity testing. Besides the fact that three *in* vitro techniques have currently been validated by the ECVAM, their application is focused on screening, rather than replacement of one of the *in* vivo studies, due to some disadvantages. The zebrafish embryo is a promising model for teratogenicity screening but its use is limited. The main issue in the current zebrafish embryo assay is the immaturity of some of the CYPs, which leads to an ongoing discussion on the metabolic capacities of the developing embryos. This is especially important for proteratogens, which might be missed in zebrafish embryos due to this low CYP-mediated metabolism, leading to false negative results. Therefore, there is a need for a metabolic activation system (MAS) in this alternative assay for developmental toxicity. **We hypothesize that the implementation of a MAS in the zebrafish embryo assay will improve its predictivity, and its application in a pharmaceutical setting**

To fulfil this hypothesis, the main goal of this doctoral project was the development of a non-toxic MAS condition for zebrafish embryos.

To achieve this, several possible toxicants which are listed below were identified based on general knowledge and a thorough literature study. Next, every single possible toxicant was examined for its role in the toxicity in the mDarT and if necessary, the protocol was optimized before investigating the next possible toxicant. An overview of possible causes of embryotoxicity and the approach of this doctoral thesis is shown in Figure 7:

1. Investigate the role of increased temperatures and its possible effect on zebrafish embryotoxicity.

    Zebrafish are raised between 26.5 and 28.5°C as this range is considered to be optimal for development of the embryos and larvae. However, to guarantee microsomal activity (which was believed to be optimal at approx. 38°C), coincubation of zebrafish embryos with a MAS is carried out at 32°C as an intermediate. This is a deviation from the optimal temperature range for development and therefore, temperatures higher than 28.5°C were assessed.

2. Selection of an optimal coincubation medium.

    Zebrafish embryos are reared in a zebrafish medium (e.g. ocean sea salts buffered with sodium bicarbonate in reverse osmosis water). Metabolic activation assays, on the other hand, are often performed in a potassium phosphate buffered medium. Therefore, a suitable medium has to be found for coincubation of liver microsomes and zebrafish embryos. There are two factors that are important in the selection of the coincubation medium. First of all, the medium should not

interfere with the CYP activity of the human liver microsomes and secondly, the coincubation medium must be non-toxic to zebrafish embryos.

3. Test whether reactive oxygen species are present in MAS and play a role in the embryotoxicity and if so, whether treatment with antioxidants can diminish the embryotoxicity.

During CYP activity, radicals can be produced which possibly leads to high levels of reactive oxygen species in the medium. This might impact the embryos and have an effect on the cellular or even organismal level. Therefore, the amount of ROS produced in the medium will be measured and pre-treatment with antioxidants will be explored.

4. Assess the toxicity of all components that are used in the microsomal preparation (*i.e.* medium, NADPH, protease inhibitors and so on) to identify and possibly replace the substances responsible for embryotoxicity.

In order to remediate the embryotoxicity caused by the MAS, we wanted to unravel which of the components in the MAS mixture is the (main) toxicant. Therefore, we tested all the components morphologically and excluded the factor causing toxicity to see whether the toxicity can be resolved this way. Also, if toxicity is still present it means that the microsomes themselves cause the embryotoxicity.

5. Explore the possibilities of a preincubation step of drugs with MAS followed by ultracentrifugation prior to coincubation with zebrafish embryos as a non-embryotoxic approach to the mDarT.

If the microsomes themselves are embryotoxic, they cannot be used for coincubation with zebrafish embryos. Therefore, the compound will be preincubated MAS followed by ultracentrifugation of the mixture to precipitate the microsomes. The supernatant will then be used, which includes the remainder of the parent compound and the newly formed metabolite(s). If toxicity is still present, dilution of this mixture to the point where it is no longer causing embryotoxicity will be carried out before exposing the embryos to this test solution. In the end, this will lead to a non-toxic preincubation system which represents the metabolizing aspect in the mDarT.

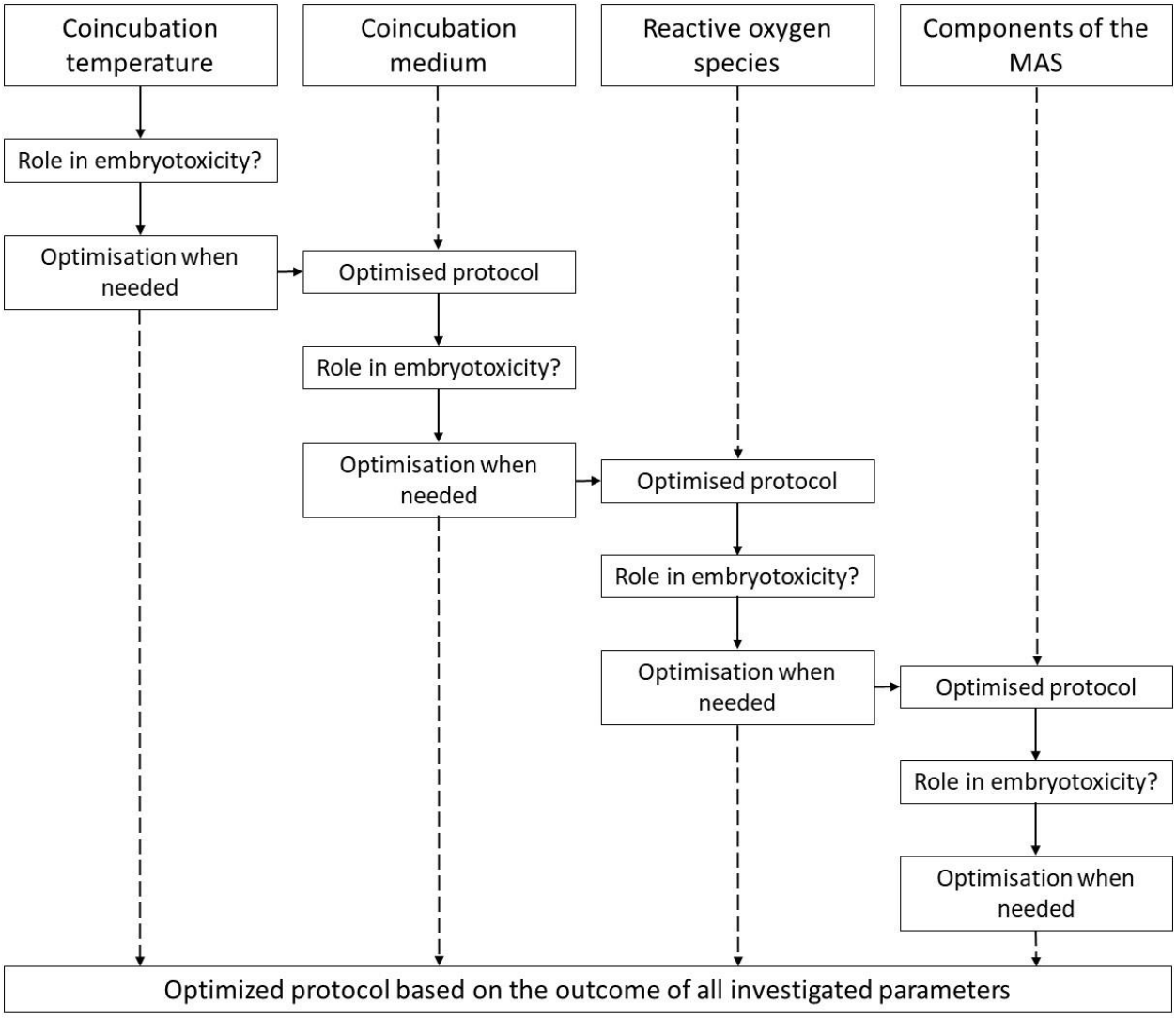

Figure 7. Workflow of the thesis. After identifying a possible involvement of a component in zebrafish embryotoxicity, the protocol was revised and the optimised protocol was used for analysis of the next component, ultimately leading to an entirely optimised protocol.

Chapter 3: Effects of different incubation temperatures on zebrafish embryonic development

Adapted from: Pype, C., Verbueken, E., Saad, M. A., Casteleyn, C. R., Van Ginneken, C. J., Knapen, D. & Van Cruchten, S. J. Incubation at 32.5°C and Above Causes Malformations in the Zebrafish Embryo. *Reprod Toxicol*. **56**, 56-63 (2015)

Abstract

Zebrafish embryos are increasingly used for developmental toxicity screening of candidate drugs and are occasionally co-incubated with a metabolic activation system at 32°C for 1, 2 or 4 h, depending on their developmental stage. As this temperature is higher than the optimal temperature for zebrafish embryonic development (26–28.5°C), we investigated whether continuous incubation of zebrafish embryos from 2.5 until 96 h post fertilization (hpf) at high temperatures (30.5–36.5°C) causes malformations. At 32.5°C tail malformations were observed as early as 24 hpf, and these became even more prominent at 34.5 and 36.5°C. Cardiovascular and head malformations, oedema and blood accumulations throughout the body were present at 36.5°C. Finally, temperatures higher than 28.5°C accelerated embryonic development except for 36.5°C, at which a lower hatching rate and hatching enzyme activity were observed. In conclusion, incubation of zebrafish embryos at 32.5°C and above from 2.5 until 96 hpf causes malformations as early as 24 hpf.

3.1 Introduction

Zebrafish (Danio rerio) embryos are currently used for acute and/or developmental toxicity testing of pharmaceuticals, chemicals and cosmetics[1-3]. So far, only the (Zebra)Fish Embryotoxicity Test ((Z)FET, OECD TG 236) has been approved by the regulatory agencies, *i.e.* to test acute toxicity of chemicals[3,4]. In this test, zebrafish embryos are exposed to the test chemical for 96 h, covering the period of organogenesis, under standardized conditions. The endpoints in this test are limited to lethality, i.e. coagulation of fertilized eggs, lack of somite formation, lack of detachment of the tail bud from the yolk sac and lack of heartbeat[3,4]. However, several research groups and pharmaceutical companies are also using the ZFET study design to assess developmental toxicity of drugs and chemicals by adding several other endpoints such as edema, blood accumulations, malformations of the head and cardiovascular abnormalities[2,5-10]. Several acronyms are used in literature to denominate this assay, such as Danio rerio Test (DarT) or zebrafish embryo developmental toxicity assay (ZEDTA)[2,5-8,10]. The latter term has been introduced in order to clearly differentiate this assay from other zebrafish embryo tests, as the DarT was originally introduced by Nagel in 2002[11] to assess acute toxicity. In 2008, Busquet et al. introduced another term, i.e. mDarT[16]. In this assay, zebrafish embryos were only briefly exposed to the test substance, i.e. from 2 until 3 hours post fertilization (hpf), but in the presence of an exogenous metabolic activation system (MAS) at 32°C. They were then reared at 26°C and morphologically evaluated until 48 hpf (figure 1). This MAS consists of induced rat liver microsomes, which contain high concentrations of cytochrome

p450 enzymes (CYPs) that are important in phase 1 metabolism of xenobiotics[17,18]. Busquet et al. developed this DarT assay with metabolic activation (mDarT) to cover biotransformation of proteratogens[15,&§]. Weigt et al.[15] initially followed this reasoning and slightly adapted this mDarT protocol by extending the rearing and evaluation period to 72 hpf (Figure 1). However, in later studies they showed that several proteratogens caused malformations in zebrafish embryos without addition of rat MAS[9,13], which could suggest that no metabolic activation system is required. The latter is still a matter of debate as some compounds, such as allyl alcohol[14], acetaminophen[15] and albendazole[10], appear not to be biotransformed by zebrafish embryos. Therefore, this has also been taken up as a potential limitation of the ZFET acute toxicity test[12]. This said, the published mDarT protocols also show limitations as induced rat MAS is toxic for the embryos and consequently only short exposure windows, not covering the entire period of organogenesis, are used in these assays[10,15,16]. Indeed, Mattsson et al.[10] showed that continuous incubation of 2 hpf zebrafish embryos with rat MAS alone caused a developmental delay after 5 h and embryonic death, which was noted after 2 days of exposure. A stepwise reduction of the MAS concentration to 1.4% of the original concentration (0.7 mg/mL microsomes; 10 mM NADPH) did not sufficiently reduce MAS toxicity to allow continuous exposure for days, starting at 2 hpf. After 24 h of exposure all embryos were still developmentally delayed. Therefore, various incubation durations were tested, starting from 2, 12 and 24 hpf. The longest tested tolerable incubation times were 1 h from 2 hpf, 2 h from 12 hpf and 4h from 24 hpf and these exposure windows were applied in their final protocol (figure 1).

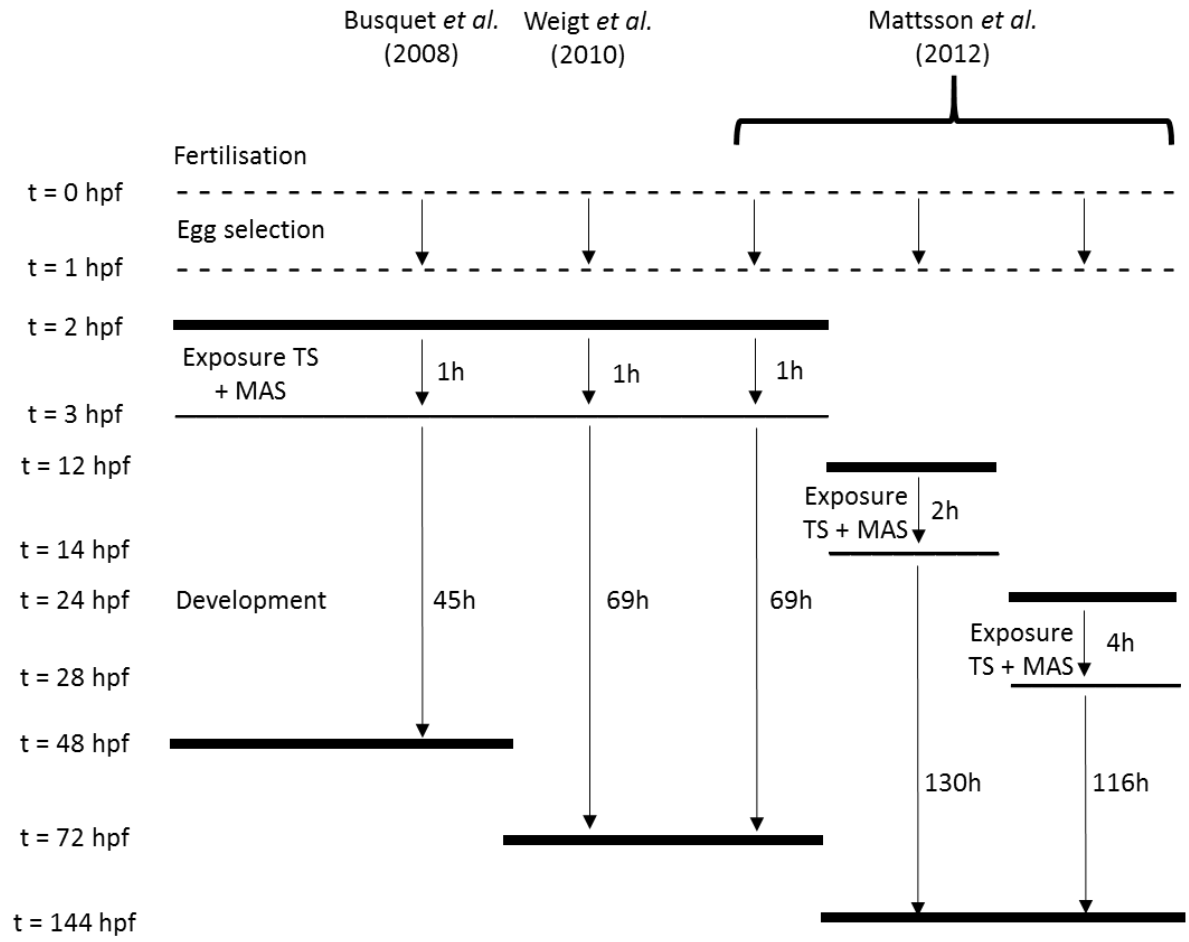

Figure 1. Overview of published mDarT protocols. The exposure of the zebrafish embryos to the test substance (TS) with induced rat MAS was performed at 32°C in all protocols. Further rearing of the embryos until 48 hpf, 72 hpf or 144 hpf was performed at 26°C in all protocols.

As a high temperature (32°C) is used when co-incubating zebrafish embryos with induced rat MAS, we wondered whether this could also be a contributing factor to the embryotoxicity that Mattsson et al. observed under continuous incubation of 2 hpf embryos, even with very low MAS concentrations[10]. Although adult zebrafish tolerate extreme water temperatures in the wild, ranging from 6°C in winter up to 38°C in summer, zebrafish have a much lower threshold for temperature fluctuations during their embryonic and larval development[19]. The optimal temperature for zebrafish embryonic development is considered to be 26 - 28.5°C[15,20-22]. A higher temperature (32°C) is applied when co-incubating zebrafish embryos with rat MAS, as mammalian microsomes are considered to function optimally at 37°C[16]. Busquet et al. chose 32°C as a compromise between optimal metabolic activation and optimal embryonic development, because Kimmel et al.[21] mentioned that temperatures above 33°C may impact embryonic

development after prolonged incubation16. However, so far no morphological characterization of this temperature has been performed under continuous incubation conditions until the end of organogenesis. Therefore, the aim of this study was to investigate whether high incubation temperatures from 2.5 until 96 hpf cause malformations in zebrafish embryos.

3.2 Materials and Methods

3.2.1 Methods

3.2.1.1 Adult zebrafish housing and egg production

Adult wild type zebrafish (Danio rerio, in house wild type AB zebrafish line), designated for breeding, were kept in a recirculating Zebtec standalone housing system (Tecniplast, Hohenpeißenberg, Germany). The water temperature was set to 28°C ± 0.2°C and the conductivity and pH of the water were 500 ± 15 µS/cm and 7.5 ± 0.3 respectively. The renewal of water was sustained at 25% of its total volume/day in order to keep ammonia, nitrite and nitrate levels below detection limits. An automated light cycle of 14/10 hours light/dark was applied.

The fish were fed thawed food (Artemia nauplii, Daphnia, Chironomidae larvae or Chaoborus larvae) twice a day, as well as granulated food (Biogran medium; Prodac International, Cittadella, Italy) at a rate of 1.5% of their mean wet weight per feeding, also twice a day.

The day before mating, one male and two female fish were transferred from the stock to separate breeding tanks. The next morning, the fish were allowed to breed and spawn during 45 minutes, after which the embryos were collected. To remove faeces and coagulated eggs, multiple washing steps in embryo solution (pH 7.5), i.e. Instant Ocean® Sea Salt (Blacksburg, VA, USA) and sodium bicarbonate (VWR, Leuven, Belgium) dissolved in reverse osmosis water (conductivity <10 µS/cm), were performed. The conductivity of the solution was set to 500 µS/cm. Within 2 hpf, all of the embryos were transferred to a 48 well plate (Greiner Bio-One, Cellstar, Wemmel, Belgium) filled with approximately 1 ml of embryo solution and checked under a Leica S8 APO microscope with Leica L2 lighting (Leica, Diegem, Belgium) for normal (i.e. no asymmetric) cell division of the first cell cycles (4- to 64-cell blastomeric stage). Unfertilized or asymmetric eggs were discarded from the experiment and replaced by other eggs.

3.2.1.2 Test conditions

Each experiment consisted of a control group (n= 96 embryos) and a test group (n=96). The controls were incubated at 28.5°C ± 0.3°C, which is the standard temperature used for rearing in our zebrafish facility. Test embryos were kept at temperatures ranging from 30.5°C up to 36.5°C with a 2°C interval (t = 30.5°C, 32.5°C, 34.5°C and 36.5°C ± 0.3°C). The selected temperature range was based on the physiological temperature of zebrafish embryos (28.5°C) and an extreme of 36.5°C, which was previously shown to cause approximately 100% embryonic death[23-25]. Control and test embryos were kept in a 14/10h light/dark cycle in a separate incubator. To avoid acidification and oxygen deprivation of the medium and to make it easier to handle the embryos during examination, 48-well instead of 96-well plates were used. At the latest at 2.5 hpf, the control and test plates with embryos were placed in their respective incubators. After 48 hpf, the medium was renewed in order to maintain high oxygen levels, reduce acidification of the medium and prevent accumulation of molecules excreted by the embryo.

In order for a batch of eggs to be valid, at least 80% of all eggs had to be fertilized and mortality of the controls had to be lower than 10% throughout the experiment. Since larvae are able to swim shortly after hatching (normally before 72 hpf and especially when manipulating them), scoring of the larvae at 96 hpf was performed under anesthesia with 0.1 g/l MS-222, pH 7.4 (ethyl 3-aminobenzoate methanesulfonate, tricaine; Sigma-Aldrich, Diegem, Belgium). At the end of the experiment, the larvae were euthanized by placing them in a 1 g/l solution of MS-222, pH 7.4. Each experiment was performed in duplicate to assess reproducibility of our results.

3.2.1.3 Morphological evaluation of the embryos

The embryos were evaluated at several time points (5.25, 10, 24, 48, 72 and 96 hpf; see table 1), which represent the transitions of one developmental stage to another according to Kimmel *et al.*[21]. Apart from coagulation, a series of morphological parameters, adapted from Nagel[11], were examined depending on the time point. An overview of all parameters is given in Table 1.

Coagulation or embryonic death was distinguished from living embryos by the turbid content of the chorion and the absence of a heartbeat. Hatching, normally occurring between 48 and 72 hpf, was scored as successful when the head of the embryo was outside the chorion. As a consequence, a non-hatched score was given when only the tail was outside this structure. Deviations of the tail included slight curves or complete bends. Edemas and blood accumulations were registered in the entire embryo, including the pericard and the head. Malformations of the cardiovascular system included absent or disturbed blood circulation in the tail and abnormal looping of the heart. Malformation of the head included

malformations of the ear (e.g. number of otoliths and position of the ear), eye and mouth, whereas malformations of the pectoral fin comprised missing or deformed fins. All parameters were scored 0 if normal and 1 if a malformation was present. This binary scale was used due to the high number of analyzed parameters. As such, only a distinction had to be made between normal and abnormal development and no gradation of the severity of a malformation had to be made.

Table 1. Coagulation and morphological parameters scored at the different time points.

	Stage (hpf)					
	5.25	10	24	48	72	96
Coagulation	X	X	X	X	X	X
Hatching				X	X	X
Tail deviation			X	X	X	X
Oedema			X	X	X	X
Blood accumulation			X	X	X	X
Malformation of the cardiovascular system			X	X	X	X
Malformation of the head			X	X	X	X
Malformation of the pectoral fins					X	X

hpf = hours post fertilization

3.2.1.4 Cathepsin L assay

In order to hatch properly, embryos need to have reached the stage at which the hatching gland releases enzymes that break down the chorion[26]. As hatching was impaired at 36.5°C (see section 3.2), we evaluated whether this could be related to a loss of hatching enzyme release. Therefore, we assessed cathepsin L activity, which is an enzyme that is highly expressed in the hatching gland[27]. Cathepsin L cleaves the substrate z-Phe-Arg-7-amido-4-methylcoumarin into the fluorescent product 7-amido-4-methylcoumarin (AMC). The protocol was adapted from Trikić *et al.*[27] and 2 biological replicates were performed. Single embryos were placed into a 96-well plate in 110 µl of embryo solution at 28.5°C (n = 16) and at 36.5°C (n = 24). We included more embryos in the test group (36.5°C) than in the controls (28.5°C) to compensate for the possible loss of embryos (coagulation) at 36.5°C. At 48 and 72 hpf, 100 µl of the medium was collected and replaced with new medium. The collected medium was transferred to dark 96-well plates. To each well, 50 µl of assay buffer (340 mM sodium acetate, 60 mM acetic acid, 4 mM EDTA P.H 5.5, 8 mM DTT) and 50 µl of 20 µM substrate (*i.e.* 5 µM as final concentration) was added and

immediately placed in a Tecan Infinite M200 Pro (Tecan, Männedorf, Switzerland) after which the signal (excitation of 370 nm, emission of 460 nm) was measured every 5 minutes during half an hour.

3.2.2 Calculated data and statistical analysis

The number of coagulated embryos in the different temperature groups was used to calculate the survival rates of all six developmental stages. To determine the general developmental speed of the embryos at different incubation temperatures, hatching was reported from 48 hpf onwards and only living embryos were included in the analysis. From 24 hpf, all malformations (except malformations of the pectoral fins, which can only be evaluated from 72 hpf onwards) within a single embryo were pooled to calculate the percentage of malformed embryos per group. Additionally, the percentage of embryos showing no, one or several malformations or death was calculated for the different test groups as well. Moreover, the incidence of each scored malformation at 96 hpf was reported for the test groups and their respective controls to present an overview of the temperature induced malformations at the end of organogenesis. To assess the reproducibility of our results, the two replicates were analyzed with a chi^2-test (IBM SPSS Statistics v.22, Brussels, Belgium). SPSS was also used to analyze the percentage of embryos showing no, one or several malformations in each test group compared with their controls by means of a Mann-Whitney U test. For our binary data (i.e. 0 or 1), using binary logistic regression analysis was not possible due to so-called “zero cell count” of several parameters (i.e. very rare malformations leading to zero affected controls, or when all embryos of a certain group are affected). Therefore, odds ratios (OR) were calculated (MedCalc v. 13.1., Ostend, Belgium) with a Haldane correction, where needed. The latter means that a value of 0.5 is added to each cell with a zero-cell count. With a z-test, the p-value indicates whether the odds are significantly different between controls and the test group. For the cathepsin L data, we compared the group of embryos reared at 28.5°C with those reared at 36.5°C at 48 and 72 hpf using the Independent Samples T-Test (IBM, Belgium). Outcomes were considered significant when the p-value was ≤ 0.05.

3.3 Results

3.3.1 Validity and reproducibility of the experiments

All batches of eggs were valid as the mortality in all control groups never exceeded 10% and the overall percentage of control embryos showing malformations was less than 5%. The results of the experiments were reproducible as no statistical difference for any of the parameters was present between the two replicates. Only for hatching at 48 hpf a difference between the two replicates was noted ($p < 0.05$). However, hatching is known to be a very variable parameter, normally occurring between 48 hpf and 72 hpf[21]. Therefore, all values reported in this paper were taken arbitrarily from the first replicate.

3.3.2 Coagulation, hatching and total number of malformed embryos

Coagulation, or embryonic death, occurred only in the highest temperature groups (34.5°C and 36.5°C) and late during development (figure 2). At 34.5°C and 96 hpf, coagulation was significantly higher ($p = 0.0014$) than the controls. Embryos incubated at 36.5°C had a 12.2 ($p < 0.0001$) and 55 ($p < 0.0001$) times higher chance to coagulate at 72 and 96 hpf, respectively. At 36.5°C, this resulted in a 74% chance that the embryos/larvae would coagulate whilst incubated at this temperature.

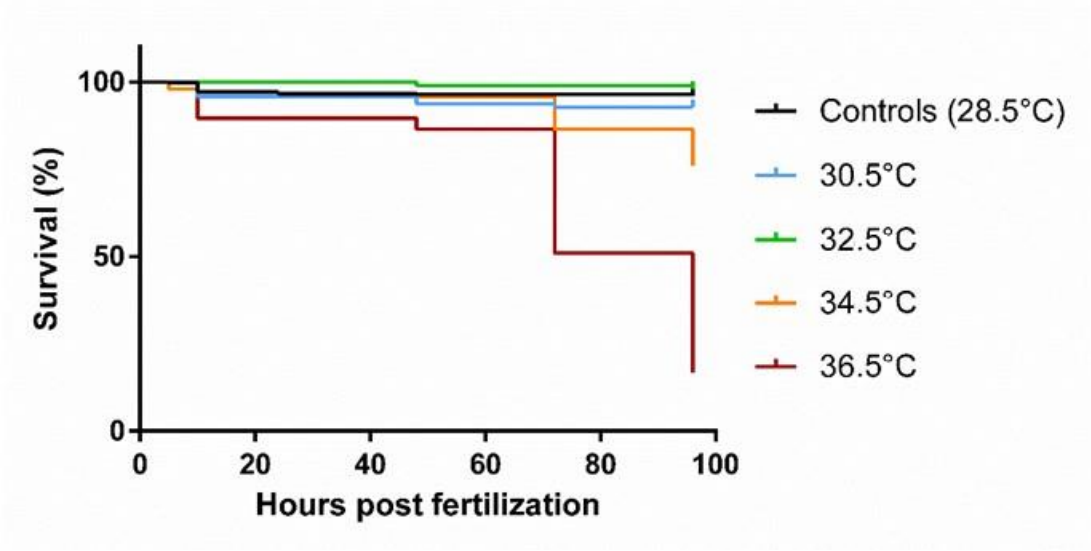

Figure 2. Survival curve for the pooled controls and all tested temperatures.

Embryos incubated at 30.5°C, 32.5°C and 34.5°C showed a significantly higher chance of hatching compared to the controls (OR = 0.08, 0.005 and 0.23, respectively, $p \leq 0.0001$). At 36.5°C, the opposite effect was noted (OR = 19.72, $p = 0.0411$). In figure 3, this is visually represented as the percentage of embryos that hatched and did not hatch at 48 hpf in the different test groups.

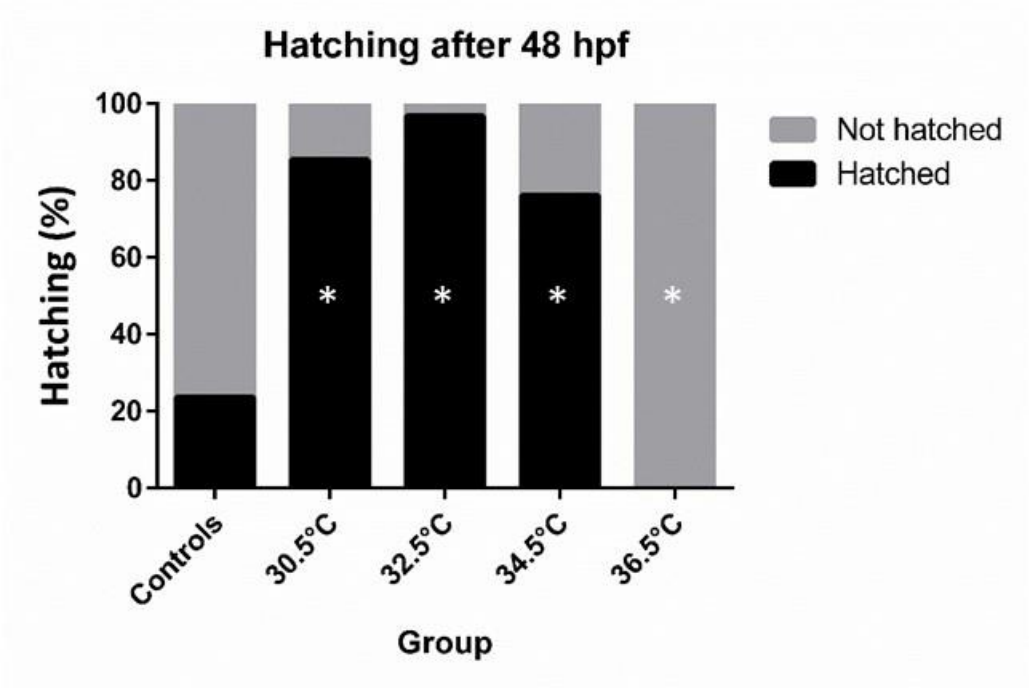

Figure 3. Proportions of hatched embryos at 48 hpf for the pooled controls and the different test groups. All test groups were significantly different from their respective controls, as indicated by the white asterisks (*). P-values for 30.5°C – 34.50°C ≤ 0.0001 and 0.0411 for 36.5°C. From 30.5 - 34.5°C, there was an increase of hatched embryos, whereas there was a decrease of already hatched embryos at 36.5°C.

At 36.5°C, most embryos also failed to hatch at 72 hpf (OR = 150.27; $p < 0.0001$) and at 96 hpf (OR = 285.92; $p = 0.0002$). Often embryos remained with their heads and upper bodies in the chorion, whereas the tails were partially out of the chorion (see figure 6D). Additionally, embryos that were not hatched at 72 hpf had a 4.35 fold ($p = 0.0477$) higher chance of being coagulated at 96 hpf. This could not be explained or predicted by morphological effects at earlier time points as non-hatched embryos at 72 hpf showed a similar incidence and type of malformations at 48 hpf and 72 hpf compared with the embryos that did hatch at 72 hpf.

Regarding the total number of affected embryos, no significant increase was found at 30.5°C compared to the controls. However, at 32.5°C these numbers significantly increased as early as 24 hpf, up until the end of the experiment (OR ≥ 5.68; $p \leq 0.0024$) (Figure 4B). In the highest temperature groups (34.5°C and 36.5°C) this observation was even more distinct, with ORs for the different time points (48 - 96 hpf) of OR34.5°C ≥ 11.55, $p \leq 0.0001$ and OR36.5°C ≥ 56.62, $p \leq 0.0001$, respectively (figure 4C and 4D). At 34.5°C, the amount of embryos having at least one malformation appeared to decrease over time. This could be linked to one parameter, i.e. edema. At 24 hpf, the number of recorded edemas (head, yolk and pericard)

was 73, whereas the numbers of edema that were observed at 48, 72 and 96 hpf were 35, 27 and 7, respectively. At 36.5°C, the amount of embryos that was impacted was already high at 24 hpf and remained high at the later time points (figure 4D).

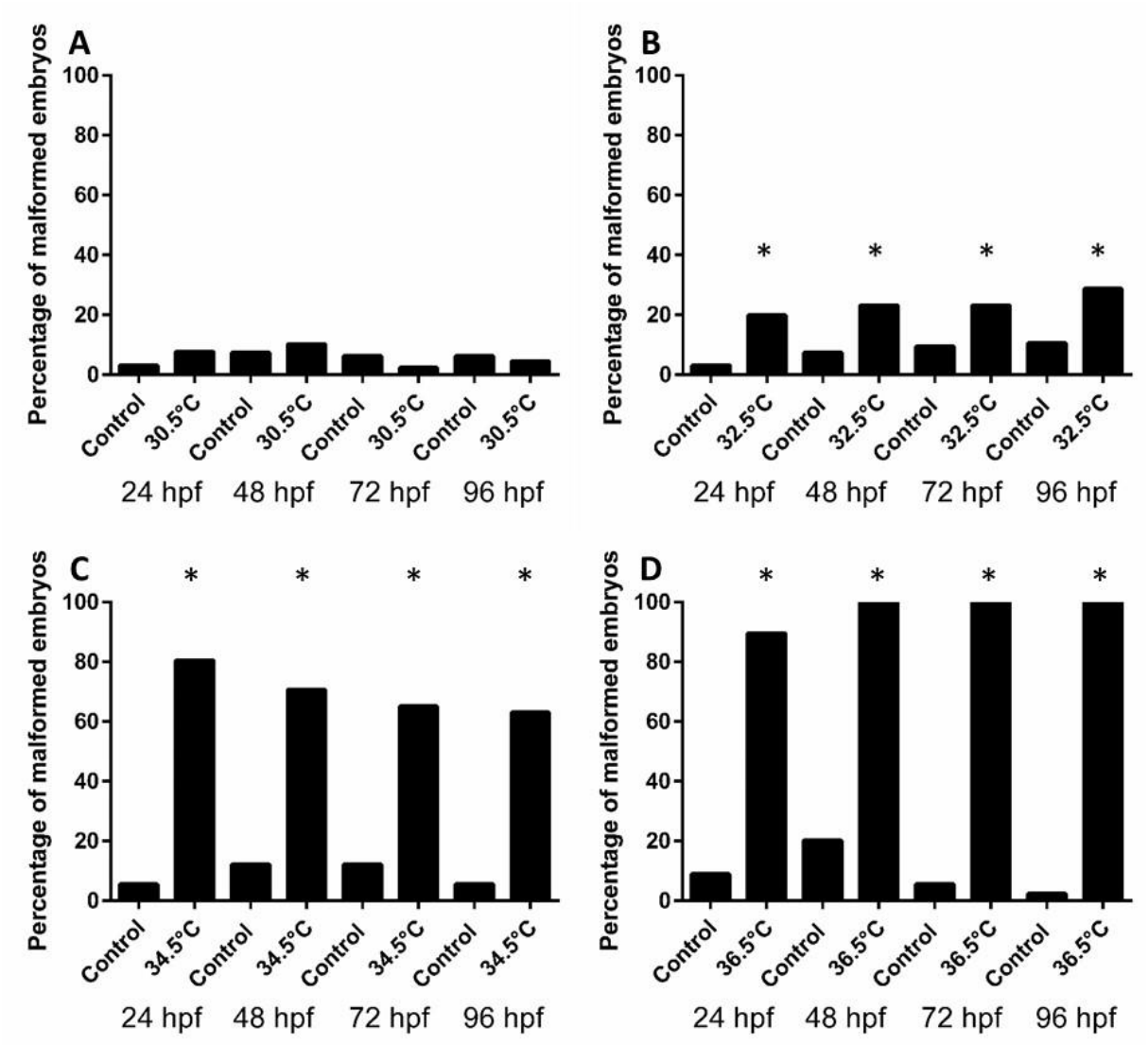

Figure 4. Representation of the percentages of embryos having at least one malformation at the different time points. A. 30.5°C, B. 32.5°C, C. 34.5°C and D. 36.5°C. Statistical differences compared to their respective controls (28.5°C) are indicated with asterisks (*).

Regarding the average number of malformations per embryo, the analysis yielded results that were in line with the above-mentioned results. At 30.5°C, no difference was evident at any of the time points ($p \geq 0.173$). For temperatures of 32.5°C and above, there was a statistically significant higher amount of

malformations per embryo from 24 hpf onwards ($p \leq 0.012$). For 34.5 and 36.5°C, a proportional increase in the amount of malformations with increasing temperature was seen ($p \leq 0.004$). Results at 96 hpf are shown in table 4.

Table 4. Representation of the percentages of embryos for each test group having one or more malformations at 96 hpf.

Temperature	Number of malformations per embryo								p-value
	0	1-2	3-4	5-6	7-8	9-10	11-12	Unscored*	
30.5°C	88.5	4.2	0.0	0.0	0.0	0.0	0.0	7.3	< 0.001
32.5°C	69.8	19.8	4.2	1.0	2.1	0.0	1.0	2.1	< 0.001
34.5°C	28.1	35.4	2.1	3.1	0.0	1.0	0.0	30.2	0.002
36.5°C	0.0	2.1	1.0	2.1	8.3	1.0	2.1	83.3	0.577

*Unscored embryos are either coagulated or their body parts were too malformed to score the embryo.

3.3 Detailed evaluation of the surviving embryos at 96 hpf

Figure 5 gives an overview of the percentage of malformations for all scored parameters at 96 hpf in all temperature groups. Embryos incubated at 30.5°C barely showed malformations. At 32.5°C, one parameter was affected, *i.e.* 21.51 % of the embryos showed a tail malformation (figure 6B). The incidence of this malformation increased with higher temperatures. At 34.5°C, tail malformation was still the only parameter that was significantly more abundant in the test group. It should however be noted that some of the other parameters showed a tendency towards statistical significance (e.g. edema pericard (6.85 %), malformation of the cardiovascular system (5.48 %), malformation of the head (6.85 %) and blood accumulation in the heart (6.85 %)). At the highest temperature (36.5°C) almost all parameters were affected (figure 5). So, not only did the embryos die (Figure 2), all surviving embryos suffered from a malformation and the amount of malformations per embryo was also higher than in the lower temperature groups (figures 5 and 6).

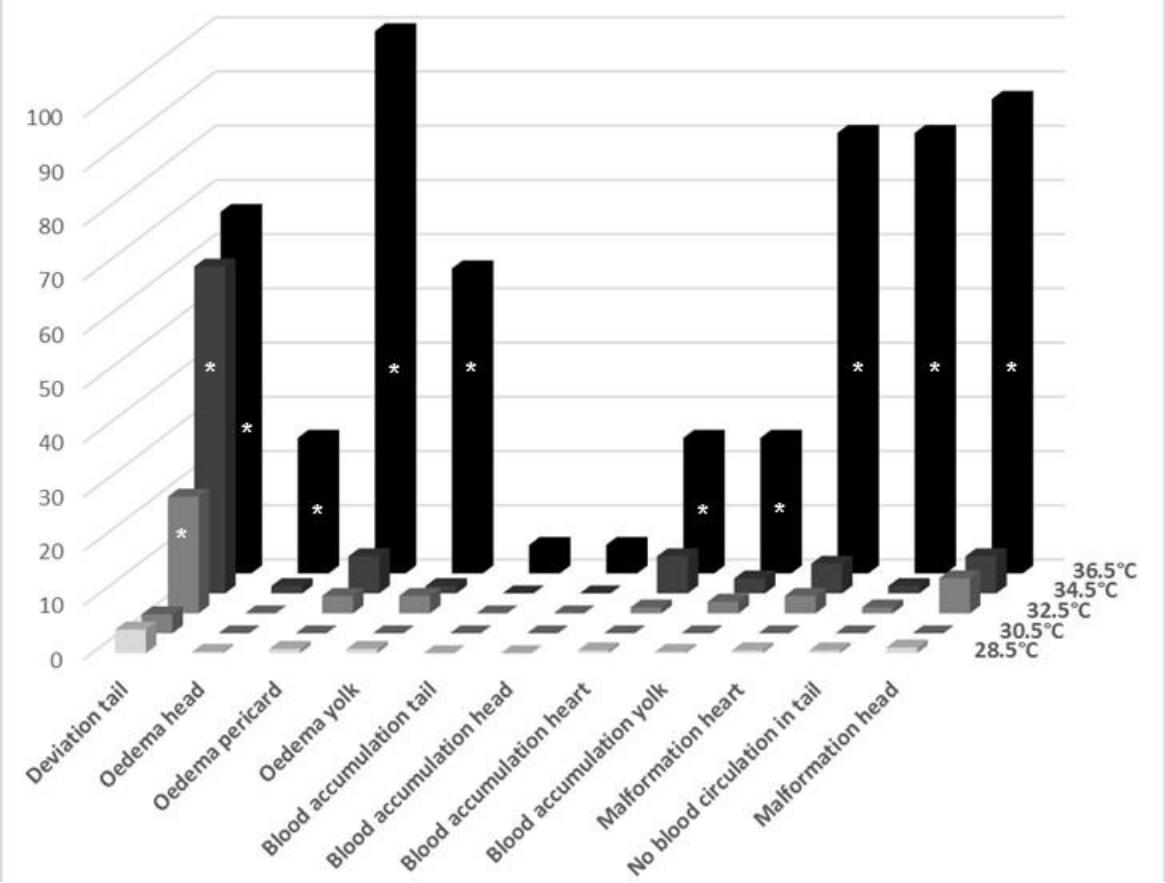

Figure 5. Percentage of embryos having a specific malformation for all temperature groups. Significant differences of the test groups compared to the controls are indicated with white asterisks (*).

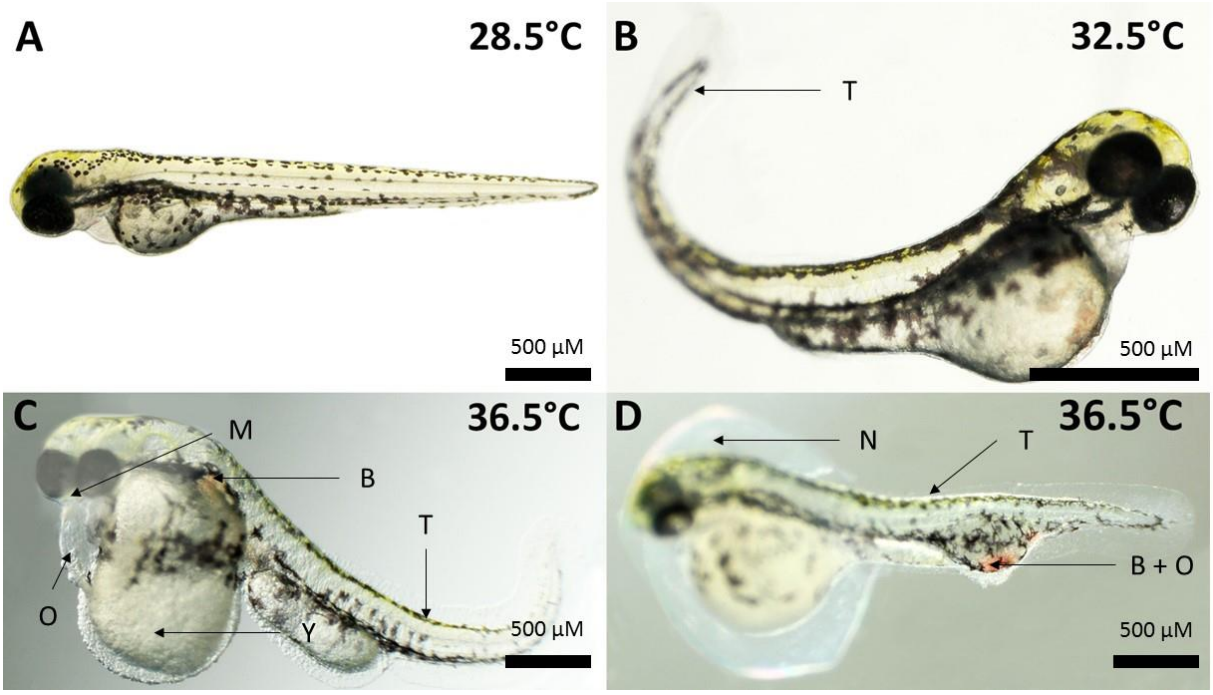

Figure 6. Overview of several observed malformations. A. Normally developed embryo (96 hpf). B. Typical example of an embryo reared at 32.5°C showing a tail malformation (72 hpf). C & D. Embryos reared at the highest temperature showing several malformations (96 hpf). Abbreviations: B: Blood accumulation, M: Malformation mouth (underdeveloped), N: Non-hatching, O: Edema, T: Tail malformation (bended tail), Y: Malformation yolk.

3.3.4 Cathepsin L assay

Cathepsin L activity (figure 7) was higher at 48 hpf for the embryos incubated at 28.5°C (controls) compared to 36.5°C in both replicates ($p = 0.0005$ and 0.006, respectively). At 72 hpf, no difference in cathepsin L activity was noted between controls and 36.5°C in the first replicate ($p = 0.397$). However, the cathepsin L activity was still higher in the controls than at 36.5°C in the second replicate ($p = 0.025$).

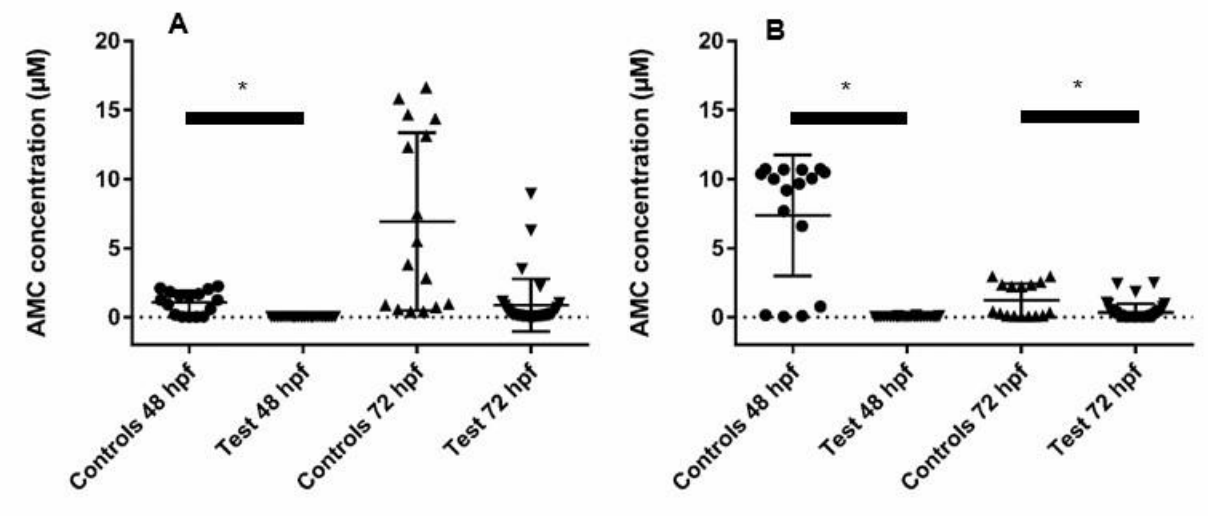

Figure 7. Cathepsin L activity (expressed as µM of AMC formed after 30 minutes) for the control (28.5°C) and test (36.5°C) groups at 48 and 72 hpf, respectively. A. First replicate. B. Second replicate. Statistical differences between control groups and test groups are indicated by bars with asterisks (*) above them.

3.4 Discussion

Heat is a well-known teratogen in a wide variety of vertebrates, and biota in general[29-31]. The observed effects range from embryonic death to malformations and are heavily dependent on the degree, duration and timing of the exposure. This has also been shown for the zebrafish as several studies already showed embryonic death from 34°C onwards, with reported survival rates of only 43 to 45%[23,24]. In our study, in which we focused on malformations, the temperature threshold appeared to be even lower, i.e. near 32°C. This was evidenced by the high percentage of embryos (ca. 30%) that showed tail malformations, which appear to be a sensitive marker for teratogenicity in the zebrafish as this has also been reported for several other toxicants[32-37]. This seems to be in contrast with data from Kimmel *et al.* and Schirone and Gross, who described that embryos survive normally between 25 and 33°C[21,25]. However, their conclusion was based on studying embryos that were only evaluated until 9 hpf[25]. As mentioned before, duration and timing are also very important factors for teratogenicity. At 9 hpf, only a very limited part of organogenesis (5 ¼ to 96 hpf) is covered and the evaluation of these embryos is restricted to developmental progression and survival. Malformations of the tail, cardiovascular system, head and pectoral fins and edema, and blood accumulation can only be assessed from 24 hpf onwards. So, as Schirone and Gross did not expose or evaluate embryos during organogenesis, possible effects may have been missed. This is further substantiated by a study on muscle function in the zebrafish. In this study,

zebrafish embryos were incubated at 32°C until 120 hpf and then reared at 27°C until the age of 8-9 months. Interestingly, these embryos had a lower body weight and a smaller size when reaching adulthood compared to embryos that were kept at 27°C until 120 hpf. Persistent effects on metabolic enzymes of the muscle of these zebrafish were still present when reaching adulthood[23]. In a similar study, performed earlier, shifts in the proportions of the muscle fibers, and thus the muscle phenotype, had been examined and the effects also remained present in the adult fish[24]. Although a morphological scoring of the embryos was not performed in these studies, they substantiate our findings that embryos do not tolerate 32-32.5°C and that the threshold of a teratogenic effect by increased temperature is even lower than previously suggested[25].

Increased temperatures also accelerate zebrafish embryonic development[25]. Previous studies reported a faster development at 30°C than at 26-28°C and the fastest development was observed at 32°C and 34°C[23-25]. These findings are in full accordance with our results. However, there was a sharp cut-off at 36.5°C as most embryos did not hatch at this time point. In addition, our study showed that non-hatching at 72 hpf was a strong indicator for mortality at 96 hpf in this temperature group. This is in line with the general principles of physiology, in which altered temperatures can push organisms from a homeostatic condition to compensatory mechanisms at first (such as a higher metabolic rate), which may breakdown at a certain threshold and further lead to failure, inducing permanent effects or even death. We already showed this previously in adult zebrafish[38]. However, in our current study non-hatching of embryos at 36.5°C could not be explained by the presence of specific or more malformations in these embryos at earlier time points, when compared to the minority of embryos that did hatch at 36.5°C. Therefore, we investigated the possible role of the hatching gland and noted a lower hatching enzyme activity at 36.5°C, which may explain the low hatching rate in this temperature group compared to controls (28.5°C). Variability between the two replicates was noted but at 48 hpf both replicates showed a significantly lower cathepsin L activity at 36.5°C compared to controls. Variability in timing of release of the hatching enzymes and consequently of hatching itself under control conditions has been reported previously[27]. Therefore, it is not surprising to see this variability in our replicates as well.

Based on the above, it appears that zebrafish embryos, in contrast to adult zebrafish, have a low upper temperature threshold, which is situated at less than 4°C above the upper limit of optimal developmental temperature (28.5°C).

3.5 Conclusion

Incubation of zebrafish embryos at 32.5°C and above from 2.5 until 96 hpf causes malformations as early as 24 hpf. From 4 degrees centigrade above the normal developmental temperature, the total number of affected embryos is significantly higher than at 28.5°C and these embryos mainly show tail malformations. Coincubation of embryos with a MAS at this temperature is therefore not optimal and 28.5°C should be considered for coincubation purposes.

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Chapter 4: Selection of the optimal medium for coincubation of zebrafish embryos with a metabolic activation system (MAS)

Adapted from: Pype, C., Verbueken, E., Bars, C., Van Ginneken, C.J., Knapen, D., Van Cruchten, S.J. Biotransformation Capacity and Effects on Embryonic Development of Several Incubation Media in the zebrafish developmental toxicity assay (ZEDTA).

Data in brief, in preparation

Abstract

Bioactivation of candidate drugs in the zebrafish embryo assay remains a matter of debate[1]. Bioactivation is necessary for the correct classification of proteratogens, which only exert their teratogenic effect after bioactivation to their teratogenic metabolite(s). Therefore, several research groups have started using an exogenous Metabolic Activation System (MAS) for coincubation with zebrafish embryos[2–4], which is composed of liver microsomes containing highly concentrated CYPs. It is pivotal to rear the zebrafish embryos in a non-toxic medium in which the cytochrome P450 enzymes responsible for the majority of bioactivation of drugs are still active. Thus, first, several candidate media for coincubation of zebrafish embryos with a MAS were tested for their metabolic capacity (i.e. CYP activity) at 28.5°C. CYP activity data were acquired by means of the fluorogenic ethoxyresorufin-O-deethylase assay (EROD assay). Of all media tested, only four media showed no differences in activity compared to the routinely used potassium phosphate buffer. These four media were then withheld and used as incubation media for zebrafish embryos and early larvae to examine potential morphological effects of the media on zebrafish embryonic development at 28.5°C. The potassium phosphate buffer was included as well. Scoring was carried out at several timepoints (i.e. $5^{1/4}$, 10, 24, 48, 72 and 96 hours post fertilization). Morphological scoring included coagulation or embryonic death, as well as malformations such as oedema, curved tails, and hatching. Only one medium (0.1 M Tris-HCl buffer in embryo solution) out of four media tested did not show an increase in malformations compared with controls. The three other media showed 100 % mortality after 48 hours post fertilisation already.

4.1 Introduction

Previous research in our group showed that the temperature that is applied when coincubating zebrafish with human liver microsomes as an exogenous metabolic activation system (MAS) is too high and causes tail deviations[5]. Moreover, the human liver microsomes have the same metabolic capacity (in terms of CYP activity) at 28.5°C and 37.5°C[6]. Therefore, the next step is to identify a suitable medium for co-incubation purposes. However, most media used for rearing zebrafish have potassium and/or phosphate concentrations that are significantly lower compared to media used in metabolic assays. Therefore, these media might not have sufficient ionic strength for the enzymes to be active in metabolic assays[7–9]. On the other hand, a commonly used buffer for *in* vitro drug metabolism studies, namely potassium phosphate buffer diluted in reverse osmosis water, might be lacking some necessary salts or ions (e.g. magnesium,

calcium and chloride) that are crucial for normal embryonic development. However, there is no literature in which the minimal requirements for zebrafish rearing media are described. As a result, the potassium phosphate buffered solution might not be suited for coincubation purposes.

On the other hand, the cytochrome P450 enzymes need to be active in the selected medium, as bioactivation is required. Some critical factors play a role in enzymatic activity. These are the pH of the medium, the temperature of the solution, necessary cofactors and their concentration but also the ionic strength of the medium/buffer. Therefore, the purpose of this study was to identify several media that do not interfere with the CYP activity, whilst not causing any embryotoxicity to the zebrafish embryos. To do so, we firstly performed the fluorogenic EROD assay and selected media (*i.e.* media that did not alter CYP activity) were incubated with zebrafish embryos. The EROD assay emits a fluorescent signal after the substrate (ethoxyresorufin) is biotransformed to its product (resorufin). Thus, the fluorescent signal is a measure of activity. Hereafter morphological analysis of the media showing CYP activity was carried out.

4.2 Materials and Methods

4.2.1 Materials

Potassium phosphate buffer (0.5M) was purchased from Corning (Lasne, Belgium). Tris-HCl (1M) and both Hanks' balanced salt solutions (HBSS) were purchased from Thermo Fisher (MA, USA) and Instant Ocean sea salt from Instant Ocean (Blacksburg, VA, USA). Physiological water (0,9% NaCl) was purchased from Braun (Melsungen, Germany). $CaCl_2.2H_2O$ and $NaHCO_3$, as well as 3-(N-morpholino)propanesulfonic acid (MOPS) was purchased from Sigma-Aldrich (Diegem, Belgium) and KCl and $MgSO_4.7H_2O$ were purchased from VWR (Leuven, Belgium) and UCB Pharma (Leuven Belgium), respectively. Human liver microsomes (pooled, 50 donors, Lot PL050B) were purchased from Thermo Fischer and the NADPH regenerative system solution A and B were purchased from Corning. Ethoxyresorufin and resorufin were purchased from Sigma-Aldrich. Black 96-well plates (chimney) for the EROD analysis and transparent 48-well plates (chimney) for the morphological scoring were purchased from Greiner Bio-One (Wemmel, Belgium).

4.2.2 CYP activity assay

The ethoxyresorfin-O-deethylase assay (EROD assay) was performed at 28.5°C with a human liver microsomes concentration of 200 µg/ml and an ethoxyresorufin concentration of 10 µM. The cofactor NADPH was added as a regenerative system consisting out of two solutions (named by the supplier as

solution a and b) which were added as 5 and 1%$_{v/v)}$, respectively. These products were dissolved in the media listed below (table 1).

Table 1. Overview of different media tested

Medium	**Remarks**
0.1M Potassium phosphate buffered medium (PPBM)	0.1M of phosphate buffer with RO-water as solvent
Instant Ocean sea salts	Salts added to RO-water until a conductivity of 500 µS/cm was reached
0.1M PPBM in Instant Ocean sea slats	0.1M of phosphate buffer and 500 µS/cm of sea salts in RO-water
Hanks' balanced salt solution (HBSS)	Prepared according to Westerfield, 2007[10]
HBSS with calcium and magnesium	Purchased from Gibco
HBSS without calcium and magnesium	Purchased from Gibco
Instant Ocean sea salts buffered with 3-(N-morpholino)propanesulfonic acid (MOPS)	MOPS purchased from Sigma-Aldrich
0.1M PPBM in embryo solution	0.1M of phosphate buffer with embryo solution* as solvent
0.1M Tris-HCl	0.1M of Tris HCl with RO-water as solvent
0.3M Tris-HCl	0.3M of Tris HCl with RO-water as solvent
0.5M Tris-HCl	0.5M of Tris HCl with RO-water as solvent
0.7M Tris-HCl	0.7M of Tris HCl with RO-water as solvent
1M Tris-HCl	1M of Tris HCl with RO-water as solvent
0.01M PPBM in embryo solution	0.01M PPBM with embryo solution as solvent
0.005M PPBM in embryo solution	0.005M PPBM with embryo solution as solvent
0.001M PPBM in embryo solution	0.001M PPBM with embryo solution as solvent
Sodium chloride physiological solution	Purchased from Braun
0.1M Tris-HCl in Instant Ocean sea salts	0.1M of Tris HCl with Instant Ocean sea salts (500 µS/cm) as solvent
0.1M Tris-HCl in embryo solution	0.1M of Tris HCl with embryo solution as solvent

* Embryo solution contained 0.294 g $CaCl_2.H_2O$, 0.123 g $MgSO_4.7H_2O$, 0.059 g $NaHCO_3$ and 0.005 g KCl dissolved in 1 litre reverse osmosis water.

During the preparation of the media, the substrate was the last product to be added, after which its transformation into resorufin was initiated. Samples were measured every 5 minutes for the duration of one hour using a Tecan Infinite M200 Pro (Tecan, Männedorf, Switzerland). Excitation and emission wavelengths were 493 nm and 522 nm, respectively. Each condition was replicated 4 times.
For the analysis, the amount of resorufin formed between two data points in the linear part of the curve was used to calculate the velocity (pmol/min/mg protein). Afterwards a Mann Witney-U test was applied to compare the velocity of the test medium to the PPBM (Graphpad Prism 7, CA, USA).

4.2.3 Morphological examination

Media with an activity that was not statistically different from the control medium (PPBM) were analysed for their effects on the development of zebrafish embryos. Therefore, media were prepared as mentioned above (table 1). However, zebrafish were solely exposed to the medium and not to the human liver microsomes, NADPH regenerative system or ethoxyresorufin, to determine the toxicity of the medium itself and not the components for co-incubation in the mDarT. Control embryos were reared in a non-toxic embryo medium (Instant Ocean sea salt in RO-water, 500 µS/cm, pH 7.4). An adult zebrafish (*Danio rerio*) breeding stock was used (50 fish, random male/female distribution) and transferred to a net the day before egg collection. After 45 minutes of spawning, eggs were collected from the bottom of the tank and faeces and unfertilised eggs were removed. Eggs were subsequently divided over the 48-well plates and symmetry of the first few cell cycles was checked. Any irregular eggs were replaced with new ones. Within 2 hpf the test medium was administered to the embryos and the plates were placed in an incubator with a 14/10 hours light/dark cycle and temperature control (28.5 ± 0.3°C). Scoring was carried out at $5^{1/4}$, 10, 24, 48, 72 and 96 hpf as described earlier[11]. Two replicates of 48 embryos per replicate were carried out and the results were analysed with Graphpad Prism 7. Because the expected frequencies in the contingency tables were too low (≤5), Chi-squared analysis of the data was impossible. Therefore, we analysed the data by means of the non-parametric Fisher's exact test.

4.3 Results

Figure 1 represents the cytochrome P450 activity data of all media tested. Four media did not differ from the standard medium which was 0.1M of potassium phosphate buffered medium (PPBM) with reverse osmosis water (RO-water) as a solvent as all p-values ≥ 0.1366 (fig 1a). These media consisted of either potassium phosphate or Tris-HCl as buffer for the solution. All other media showed less resorufin formation (and thus lower biotransformation capacity) compared to the standard (p-values ≤ 0.0001). Seven of those media showed a moderate amount of resorufin formation (fig 1b), whilst seven other media had a negligible level of biotransformation capacity (fig 1c).

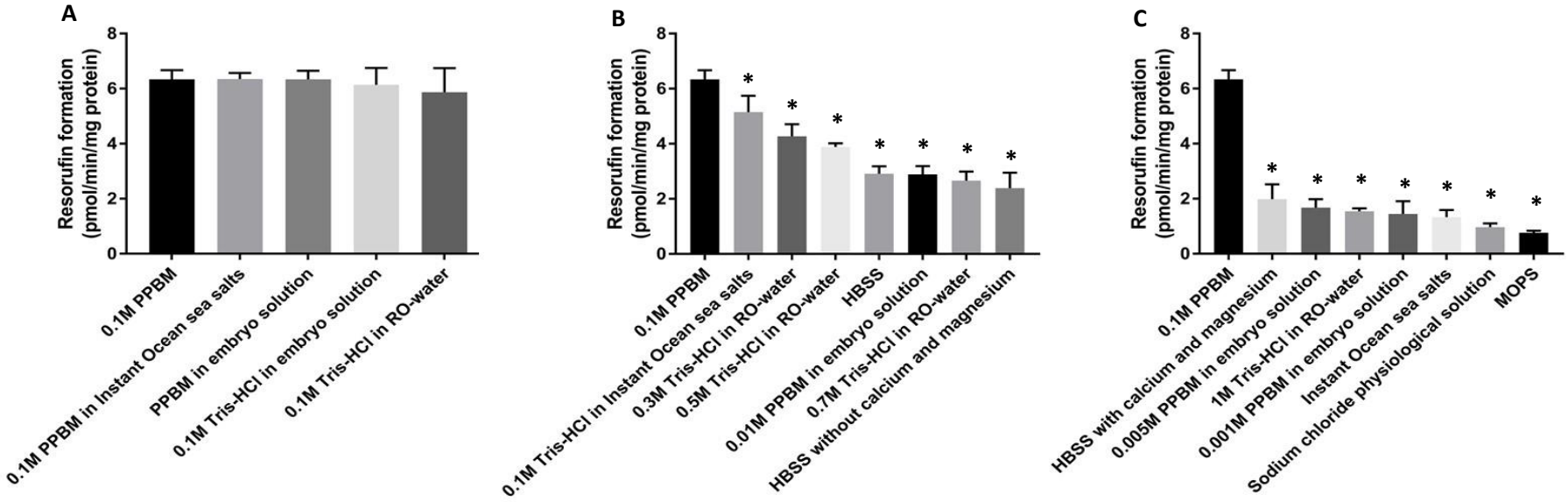

Figure 1. Resorufin formation of all media tested compared to the standard (0.1M PPBM in RO-water). **A**. Media showing no difference in resorufin formation compared to the standard. **B**. Media with a significantly lower, but moderate velocity of resorufin formation compared to the standard. **C**. Media with a negligible velocity of resorufin formation compared to the standard. PPBM: potassium phosphate buffered medium, RO-water: reverse osmosis water, HBSS: Hanks' balanced salt solution, MOPS: 3-(N-morpholino)propanesulfonic acid. * Indicate significantly lower resorufin formation compared to the controls.

The groups that did not significantly differ from the standard, as well as the standard itself, were subsequently morphologically analysed to see whether they impact normal zebrafish embryonic development by causing any embryonic death of malformations. Survival of the embryos was affected for all groups compared to the controls except for the group of embryos reared in 0.1M Tris-HCl in embryo solution ($p > 0.999$). Already after 24 hpf, an increase in lethality occurred in these groups ($p < 0.0001$) and became more pronounced with progressing duration of the experiment. For the controls and 0.1M Tris-HCl in embryo solution, coagulation was as low as 4% at the end of organogenesis (96 hpf), whilst all embryos died in every other group (figure 2).

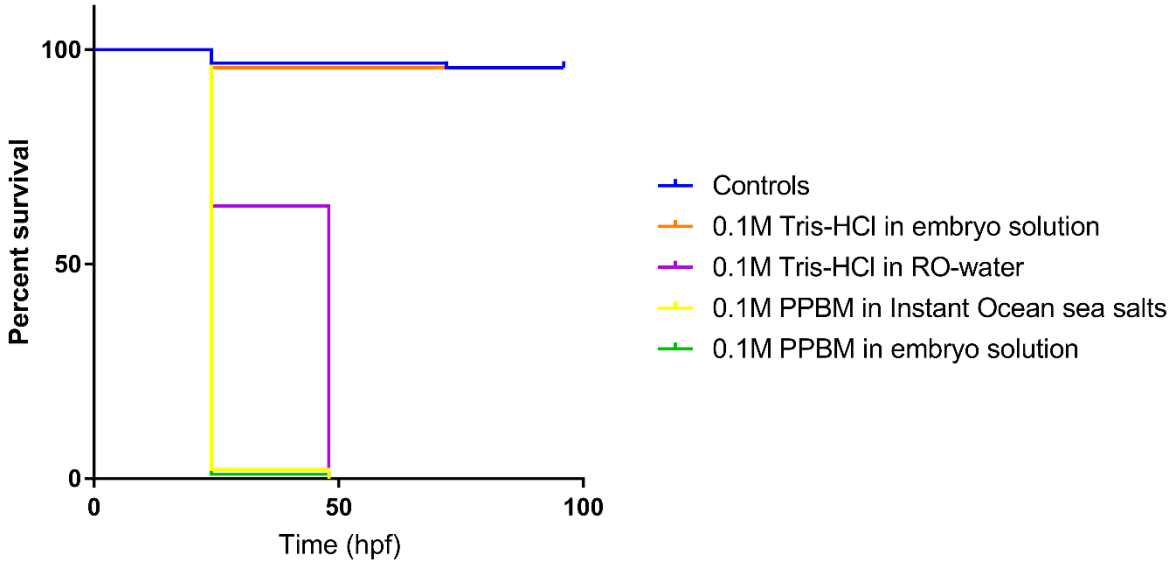

Figure 2. Survival curve of the media tested. 0.1M Tris-HCL in embryo solution did not significantly differ from the controls. All other groups differed significantly from the controls from 24 hours post fertilisation (hpf) onwards and throughout.

Because there was no increased coagulation for 0.1M Tris-HCl in embryo solution, we subsequently analysed the other morphological parameters. Results for all parameters that were affected in either one of the groups are shown in table 1 and demonstrate that there was no difference in malformations between this group and the control embryos.

Table 2. Overview of all malformation in the control group and/or 0.1M Tris-HCl in embryo solution group

Malformation	Prevalence		p-value
	Controls	0.1M Tris-HCl in embryo solution	
Coagulation	4.2%	4.2%	>0.9999
Affected embryos	3.3%	4.3%	>0.9999
Nod in the tail	2.2%	3.3%	>0.9999
Curve in the tail	2.2%	3.3%	>0.9999
Deviating tail tissue	1.1%	0%	>0.9999
Oedema of the pericardium	1.1%	1.1%	>0.9999
Oedema of the yolk	0%	2.2%	0.4973
Blood accumulation in the tail	1.1%	0%	>0.9999
Blood accumulation in the head	0%	1.1%	>0.9999
Blood accumulation in the heart	0%	1.1%	>0.9999
Disturbed blood circulation in the tail	2.2%	0%	0.4973
No blood circulation in the tail	0%	1.1%	>0.9999
Malformation of the heart	0%	1.1%	>0.9999
Deviation of the head	1.1%	1.1%	>0.9999
Deviation of the ear	1.1%	1.1%	>0.9999
Deviation of the mouth	0%	1.1%	>0.9999

Occurrence of any malformation was below 5% in both of the groups and no significant differences between the two groups could be examined.

4.4 Discussion

The results of this study showed that different types of media, of which some are used for rearing zebrafish embryos, hamper CYP activity. In a review from Bisswanger (2014)[9], some factors that are important for enzyme activity are mentioned. One of them is the pH of the medium. In our study, pH was monitored (data not shown) and within the same range for all media (*i.e.* between 7.1 - 7.8). These pH values should not interfere with liver microsomal activity, as the pH of the 0.1 PPBM medium was 7.5 and activity was high in this medium. This is not surprising, as the physiological pH for many organisms, and especially mammals is situated around a pH of 7.4. As a result, pH can be discarded as a possible cause of a decreased CYP activity.

Another factor that plays a role in enzymatic activity is temperature. Even though the temperature that was applied during the activity measurements was 28.5°C whilst the normal physiological temperature of humans is around 37.5°C, this does no alter the activity of the human liver

microsomes[6]. Moreover, all media were tested at this temperature of 28.5°C which makes that none of the media will show a difference in activity to the others due to temperature. Therefore, this factor can be ruled out. This leaves two possible criteria to have an influence on the CYP activity. One of them is the concentration of the buffer and the other is the ionic strength of the medium[9].

Concerning the concentration of the buffer, we saw that increasing the concentration of Tris-HCl did not positively increase the activity. We also tried decreasing the concentration of potassium phosphate (as it was toxic at 0.1M). However, activity decreased as well when lower concentrations of potassium phosphate were tested and a balance between no embryotoxicity and CYP activity could not be found. For the other media, concentrations were based upon other studies (which were not necessarily activity studies). For these media, it is probably the ionic strength that is the issue. As the CYP reaction is an oxidoreductase reaction dependent on heme centre containing an Fe-atom, the ionic strength of the medium might influence the redox state of Fe, possibly interfering with the enzymatic reaction[9,12]. Therefore, some buffers are better to be used in combination with CYPs than others. As a result, potassium phosphate and Tris-HCl buffered media seem to be more suitable than the other media tested.

The media that showed CYP activity were then assessed for morphological effects on embryonic development. Only one medium proved to be good, namely 0.1M Tris-HCl in embryo solution, which did not elicit any malformations or coagulation throughout the entire period of organogenesis. This was not surprising, as also other research groups are using this medium[2–4,13].

The potassium phosphate buffer was probably embryotoxic due to exuberant concentrations of either potassium, phosphate or a combination of both. As a result, 0.1M Tris-HCl buffered embryo solution is the ideal medium for coincubation purposes with MAS.

4.5 References

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Chapter 5: Antioxidants reduce reactive oxygen species but not zebrafish embryotoxicity caused by MAS

Adapted from: Pype, C., Verbueken, E., Saad, M.A., Bars, C., Van Ginneken, C.J., Knapen, D., Van Cruchten, S.J. Antioxidants reduce reactive oxygen species but not embryotoxicity in the metabolic Danio rerio test (mDarT). *Reprod Toxicol*. **72**, 62-73 (2017).

Abstract

Mammalian liver microsomes are occasionally used as a metabolic activation system (MAS) to compensate for the low CYP-mediated bioactivation of drugs in zebrafish embryos, in the so-called mDarT. However, this MAS is embryotoxic and consequently zebrafish embryos are only exposed during a very limited developmental window. The main aim of this study was to try to reduce the embryotoxic properties of MAS in order to extend the exposure window in the mDarT. Removing the microsomes from the incubation medium prior to exposure of the zebrafish embryos did not reduce embryotoxicity. Free radicals (ROS) in the incubation medium were successfully reduced by antioxidants, but the medium remained embryotoxic. Single dosing of NADPH or omitting toxic components (e.g. protease inhibitors) from the MAS preparation did also not reduce embryotoxicity. In conclusion, the exposure window in the mDarT could not be extended by reducing ROS levels, single dosing of NADPH or modifications of the MAS preparation.

5.1 Introduction

Several pharmaceutical and chemical companies are using zebrafish embryos as a screening tool for developmental toxicity[1–4] because they can be exposed and morphologically evaluated during the entire period of organogenesis (5[1/4] until 96 hpf) and as such they mimic the *in vivo* situation in mammalian developmental toxicity studies better than other alternative assays, such as the rat whole embryo culture and mouse embryonic stem cell test. However, a fundamental difference with the *in vivo* studies remains the lack of a maternal component in the alternative assays. Although most compounds are teratogenic by themselves, alternative assays could as such underestimate the teratogenic potential of drugs that would be bioactivated in a pregnant woman/dam if the enzymes that are responsible for this bioactivation are not present/still immature in the *in vitro* system. For human embryos/foetuses it has been well established that biotransformation enzymes are immature[5] and also for the zebrafish we recently showed in several studies that cytochrome P450 (CYP) activity, one of the key drivers in the bioactivation process of xenobiotics, is negligible during a major part of organogenesis[5–7]. Only at the larval stage (from 72 hpf onwards) metabolites levels above the LLOD were detected in whole body homogenates. This is also in accordance with the development of the zebrafish liver, an important drug metabolizing organ, which occurs between 72 and 96 hpf. It is in contrast though with the findings of an earlier study by Weigt *et al.* (2011), who observed malformations when exposing zebrafish embryos to several mammalian proteratogens from 2 hpf until 72 hpf[8]. However, as no metabolite concentrations were determined in that study, no conclusions could be made whether the effects were caused by the parent compound itself or by its metabolites.

So, the above clearly shows that the metabolic capacity of zebrafish embryos still remains a point of debate.

To circumvent the metabolism aspect, several research groups have been exploring the use of an exogenous Metabolism Activating System (MAS) as an add-on for the zebrafish embryo assay[9,10], in the so-called metabolic *Danio rerio* test (mDarT). However, the current mDarT protocol raises several questions, which have limited its use for developmental toxicity screening so far. First, in the mDarT assay, zebrafish embryos are only exposed to the parent compound and its metabolite(s) from 2 until 3 hpf and further reared off-dose in embryo medium until morphological analysis at 48 hpf or 72 hpf. This limited exposure window has been generally accepted as a limitation of the assay[9,10] because susceptibility to teratogens depends amongst others on exposure duration and varies with the developmental stage at the time of exposure[11]. This has been illustrated in the zebrafish for several parent compounds such as cyclophosphamide[8] and caffeine[12–14], for which no developmental defects were detected when the embryos were evaluated at 72 hpf after exposure for 1h or 24h, respectively, whereas continuous exposure to the parent compound from 2 or 5 hpf until 72 hpf clearly showed effects for both compounds. For (re)active metabolites of parent compounds the data are scarce[10,15] and to our knowledge no studies have been performed in which the morphological outcome at 72 hpf has been compared between 1h (from 2 hpf until 3 hpf) and continuous exposure (from 2 hpf until 72 hpf) to a (re)active metabolite. This said, Wilson's principles of susceptibility to teratogens apply as well to parent compounds as to their reactive metabolites, when present.

A second issue for the mDarT is the embryotoxicity caused by MAS itself during co-incubation with zebrafish embryos for longer than 1h[9,10]. It is this embryotoxicity that limits the exposure duration to the parent compound and its MAS-generated metabolites in the mDarT. So far, it remains unclear what causes the embryotoxicity during co-incubation with MAS. We previously showed that the co-incubation temperature in the mDarT, *i.e.* 32°C[9,10], is too high for normal embryonic development[16] and unnecessary for functioning of the CYP enzymes in the MAS[7], but lowering the temperature to 28.5°C did not reduce embryotoxicity in the mDarT (unpublished data). As MAS consists of mammalian liver microsomes, pieces of endoplasmic reticulum that contain CYPs in high concentrations[17], and microsomal toxicity has been reported earlier[8–10], (in)direct contact with the microsomes could be embryotoxic. Furthermore, as ROS is formed during CYP-mediated drug metabolism (see figure 1), and known to cause cell and tissue damage[18–20], ROS may also play a role in the observed embryotoxicity.

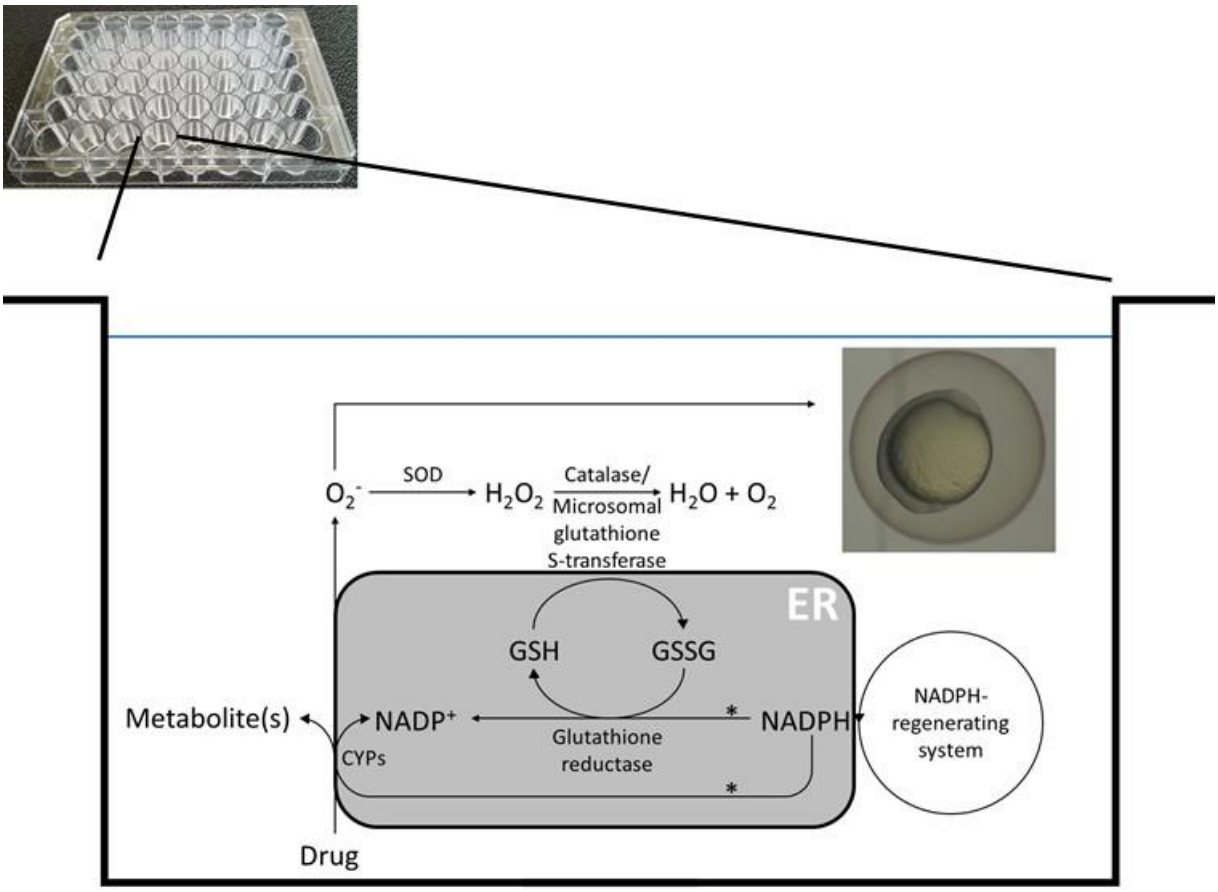

Figure 1. Simplified scheme of reactive oxygen species formation and reduction in a well of an mDarT co-incubation experiment. GSH: glutathione, GSSG: glutathione disulphide, SOD: superoxide dismutase. Grey compartment represents a microsome, which is a residual vesicle of the endoplasmic reticulum (ER). * NADPH competition. $NADP^+$ can be transformed into NADPH again in the presence of an NADPH-regenerating system (bottom right)[21–23].

In MAS, the co-factor NADPH is also required, which can be included in a microsomal setting as either a single dose of NADPH or as an NADPH-regenerating system. NADPH has a paradoxical function in terms of ROS formation/reduction in presence of microsomes (Figure 1). NADPH can be a ROS reducer in microsomes, *i.e.* as a co-factor for the reduction of GSSG to GSH, which is necessary for the elimination of hydrogen peroxide (Figure 1). In contrast NADPH can be a ROS inducer as a co-factor of CYP activity leading to lipid peroxidation[24]. Free radicals and reactive oxygen species are formed at several points in this peroxidation cascade (reviewed by Lobo *et al.*, 2010)[25]. However, the presence of drugs that undergo oxidative demethylation can also reduce NADPH-triggered lipid peroxidation, probably due to competition for the common NADPH-oxidising enzyme[26] (Figure 1). Nonetheless, free radicals are still being formed during CYP-mediated drug metabolism, as demonstrated by Wu *et al.* in 2012[27], and potentially these cause toxic effects in developing zebrafish embryos.

So, in order to answer the questions above, our study had the following objectives. First, we assessed whether exposure of zebrafish embryos to a (re)active metabolite during organogenesis showed morphological abnormalities at 72 hpf compared to a 1h exposure between 2 and 3 hpf, substantiating as such the need for a longer exposure window in the mDarT. For this purpose, we used dimethadione, the (re)active metabolite of trimethadione, and compared the effects of different concentrations of dimethadione at 72 hpf after 1h exposure (2 hpf until 3 hpf) with exposure of approx. 70h (2 hpf until 72 hpf). Second, we verified whether pre-incubation with MAS for 1h followed by incubation of zebrafish embryos in the supernatant (and no microsomes) would omit or reduce embryotoxicity. As MAS, we used commercially available human liver microsomes (pooled material from 50 donors) because they represent the best MAS for human risk assessment due to their human metabolite profile. Third, we investigated whether ROS are generated by MAS in the mDarT and if so, whether antioxidants (*i.e.* gallic acid, rosmarinic acid and selenium-methionine) reduce the amount of ROS in the medium and potentially also the embryotoxicity in the mDarT. Finally, we evaluated whether the NADPH-regenerating system or specific components of the liver microsomal preparation (*i.e.* sucrose, potassium phosphate, EDTA and protease inhibitor) influence CYP activity in the microsomes and/or cause embryotoxicity. For this latter goal, we prepared and used rat, not human, liver microsomes because manufacturers protect the exact composition of their batches and healthy human liver samples are very difficult to obtain.

Separation and purification of metabolites is expensive, labour-intensive and requires specific infrastructure. So, our ultimate goal is to develop a user-friendly complementary system for the zebrafish embryo assay that could be used in a tiered approach to expose zebrafish embryos to metabolites during the entire organogenesis according to Wilson's principles[11]. As a first step, we decided to investigate and potentially modify in this study the factors that cause the embryotoxicity of the MAS in the mDarT because it is a promising, cheap and user-friendly system. The flowchart for this experiment is visualised in figure 2.

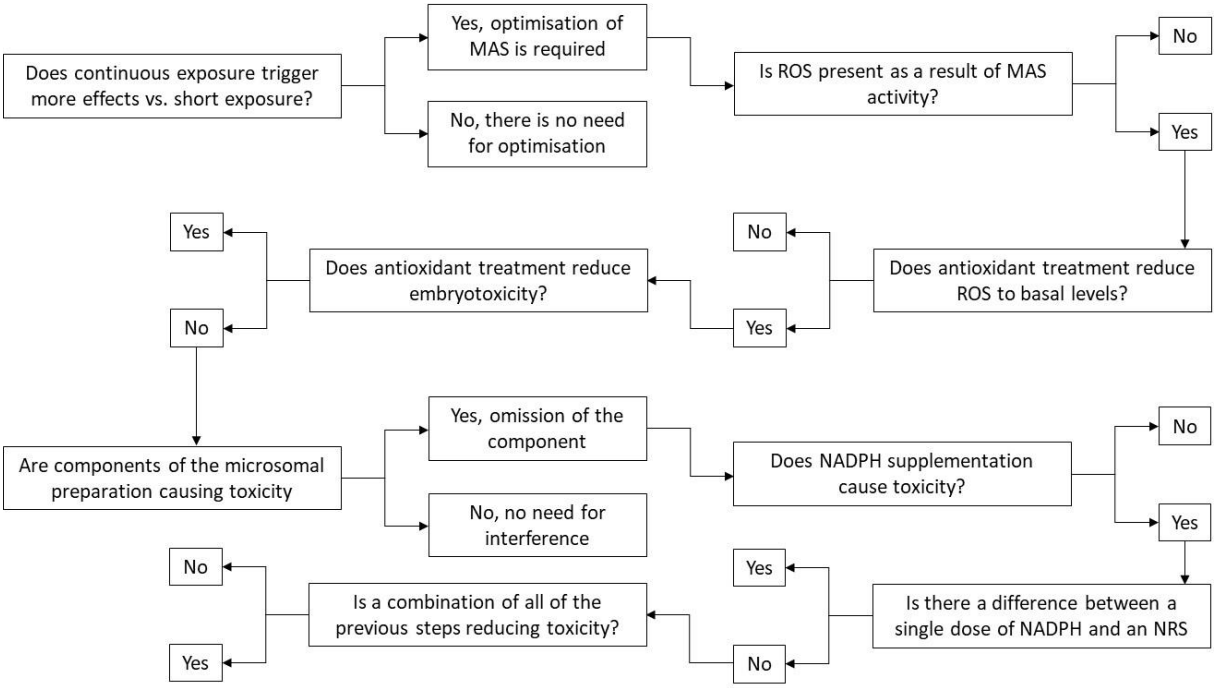

Figure 2. Flowchart of chapter 5. NRS: NADPH regenerating system

5.2 Materials and methods

5.2.1 Chemicals and products

NADPH tetrasodium salt was purchased from Roche (Vilvoorde, Belgium), Calcium chloride ($CaCl_2.2H_2O$), ethoxyresorufin, ethyl 3-aminobenzoate methanesulfonate (MS-222), gallic acid, pentobarbital, PBS, rosmarinic acid, Se-methionine and sucrose were purchased from Sigma-Aldrich (Diegem, Belgium). CM-H_2DCFDA, EDTA, Halt Protease Inhibitor Single-Use Cocktail, tris-HCl (1M, pH 7.5) and ultrapure water were purchased from Thermo Fisher Scientific (MA, USA). Potassium chloride (KCl) was purchased from Merck (Overijse, Belgium), sodium bicarbonate ($NaHCO_3$) from Acros Organics (Geel, Belgium) and magnesium sulphate ($MgSO_4.7H_2O$) from UCB Pharma (Leuven, Belgium). DMSO was purchased from Fischer Scientific (Leicestershire, UK). The human liver microsomes (pooled, 50 donors, lot PL050 B) and NADPH-regenerating system (solutions A and B) were purchased from Gibco (Thermo Fischer scientific, USA) and Corning (Lasne, Belgium), respectively. Potassium phosphate buffer and supersomes insect cell microsomes were purchased from Corning as well. Instant Ocean Sea Salt was purchased from Instant Ocean (Blacksburg, VA, USA).

5.2.2 Adult zebrafish housing and egg collection

An adult zebrafish breeding stock (*Danio rerio*, in house wild type AB zebrafish line) was maintained. The fish were kept in tanks of approx. 50 liters. The water temperature was set to 28.5 ± 0.2°C and the conductivity and pH of the water were 500 ± 25 µS/cm and 7.5 ± 0.3 with Instant Ocean sea salts and $NAHCO_3$, respectively. Water renewal was carried out when ammonia, nitrite and nitrate reached detectable levels. An automated light cycle was being used in the room with a 14/10h light/dark cycle. The fish were fed daily thawed food (*Artemia nauplii*, *Daphnia*, *Chironomidae* larvae or *Chaoborus* larvae), as well as granulated food (Biogran medium; Prodac international, Cittadella, Italy) at a rate of 2% of their mean wet weight per feeding. The Ethical Committee for Animal Experimentation from University of Antwerp (Belgium) approved the use of the animals (ECD 2015-51).

The day before mating, all fish were transferred to a net, which was placed in the tank to prevent the adults from eating the eggs. The next morning, the fish were allowed to spawn eggs and fertilise them for a period of approximately 45 minutes, after which the embryos were collected from the bottom of the tank. To remove faeces and coagulated eggs, multiple washing steps in tris-buffered embryo medium, *i.e*. 0.294 g $CaCl_2.H_2O$, 0.123 g $MgSO_4.7H_2O$, 0.059 g $NaHCO_3$, 0.005 g KCl and 0.1M tris-HCl (pH 7.5) dissolved in 1 litre reverse osmosis water, were performed. Within 2 hours post fertilisation (hpf), embryos were checked under a Leica S8 APO microscope with Leica L2 lighting (Leica, Diegem, Belgium) for normal (*i.e*. no asymmetric) cell division of the first few cell cycles (4- to 64-cell stage). Unfertilised or asymmetric eggs were discarded from the experiment and subsequently replaced by another egg. After selecting sufficient eggs, all eggs were randomly transferred to a 48 well plate (Greiner Bio-One, Wemmel, Belgium) filled with control embryo medium or one of the test solutions dissolved in embryo medium. In order for a batch of eggs to be valid, a fertilisation rate of ≥ 90% and control embryo death of ≤ 10% was necessary. All experiments fulfilled both criteria.

5.2.3 Morphological test conditions for the evaluation of embryotoxicity

Each experiment consisted of a negative control group, test groups and, if applicable(/available), a positive control group. Each experiment was replicated twice, unless 100% mortality in the test groups was observed and controls developed normally (*i.e.* ≤ 5% of embryos coagulated/malformed).

All embryos were incubated at 28.5°C ± 0.3°C with a 14/10h light/dark cycle[16]. To avoid acidification and oxygen deprivation of the embryo medium and to make it easier to handle the embryos during examination, 48-well plates instead of 96-well plates were used and every 48 hours the embryo medium or test solution was renewed[16]. A total volume of 300 µl/well was used. At the latest at 2 hpf eggs were exposed to the test solutions and at the latest at 2.5 hpf, the control and test plates were placed in the incubator.

In order for a batch of eggs to be valid for experimentation, a minimum of 80% of all eggs had to be fertilized and mortality of the controls had to be lower than 10% throughout the experiment. Since larvae are able to swim shortly after hatching (which normally occurs before 72 hpf), scoring of the larvae at 96 hpf was performed under anaesthesia with 0.1 g/l MS-222, pH 7.4. Embryos were scored as previously described[16] for several parameters including coagulation, hatching, oedema, blood circulation and tail deviations throughout organogenesis ($5^{1/4}$, 10, 24, 48, 72 and 96 hpf). At the end of the experiment, the larvae were euthanized by transferring them to an overdose of 1 g/l solution of MS-222, pH 7.4.

5.2.3.1 Morphological comparison of the effects of short and long drug exposure

We exposed zebrafish embryos to either 30 mM trimethadione (which should approximate the EC_{50} based on a study of Weigt *et al.* (2011)) for 70 hours (2-72 hpf) or its teratogenic metabolite, dimethadione (0.03, 0.3, 3 and 30 mM), for either 1 hour (2-3 hpf) or 70 hours (2-72 hpf). Controls were reared in embryo medium at 28.5°C until 72 hpf. We also included a MAS-control, *i.e.* zebrafish embryos that were co-incubated with 200 µg/ml human liver microsomes (HLM) in embryo medium for 1h, and a MAS test group in which zebrafish embryos were co-incubated with 200 µg/ml HLM and 30 mM trimethadione for 1h. All short-term exposure groups were washed after 1h of exposure and further reared in embryo medium until 72 hpf. At 72 hpf, all embryos were morphologically scored as described above. We used 20 embryos for each group and the groups exposed to the different concentrations of dimethadione were replicated.

5.2.3.2 Morphological evaluation of the toxicity of the supernatant of MAS

The toxicity of the supernatant of MAS was tested by pre-incubating human liver microsomes (200 µg/ml) and NRS (5‰$_{v/v}$ solution A and 1‰$_{v/v}$ solution B) for one hour in embryo medium at 28.5°C. Afterwards, the MAS was ultracentrifuged at 100 000 x g (4°C) for 1h and the supernatant was carefully transferred to the test embryos (n = 24) at 2 hpf. Control embryos (n=120) were reared in embryo solution for the entire duration of the experiment. The embryos were placed in the incubator and scored for lethality at $5^{1/4}$, 10, 24 and 48 hpf.

5.2.3.3 Morphological effects of gallic acid on zebrafish embryos during co-incubation with HLM

To test whether adding antioxidants is beneficial for zebrafish embryonic development by reducing ROS in the medium, we co-incubated zebrafish with HLM and the NRS in the presence and absence of an antioxidant (gallic acid) in a low and high concentration of 100 and 500 µM, respectively. Based on preliminary data[28], we used 12 embryos for every condition as all test solutions were likely to cause

100% mortality. Control embryos were reared in embryo medium (see above). A positive control, known to cause 100% lethality, was included by exposing embryos to 200 µg/ml HLM, 5% NADPH solution A and 1% NADPH solution B. Test groups were the same as the positive control but with addition of gallic acid in one of the two concentrations. We also included two groups that were solely exposed to 100 or 500 µM of gallic acid to screen for any toxic effects of the antioxidant on zebrafish embryonic development. The microsomes were pre-incubated with GA for 1h prior to co-incubation with the embryos.

5.2.3.4 Evaluation of embryotoxicity of a single dose of NADPH tetrasodium salt

NADPH as a single dose (1.25 mM) was tested for its toxicity. Therefore, a control group (n = 20) and test group (n = 20) were included. The control group consisted of embryos reared in embryo medium (see above), whilst embryos in the test group were also co-incubated with 1.25 mM of NADPH tetrasodium salt. No microsomes were added to either controls or test group, as we were only interested in the effects caused by the NADPH tetrasodium salt.

5.2.3.5 Evaluation of the toxicity of the different components of a microsomal preparation

Two experiments were designed to find the critical co-factor triggering toxicity or find possible additive effects of two or more compounds. For the first experiment, each group consisted out of 20 embryos and final concentrations of the products tested were 0.1 M potassium phosphate buffer, 250 mM sucrose, 1 mM EDTA and/or 1 unit/ 10 ml of Halt Protease Inhibitor Single-Use Cocktail (see table 1). Sucrose and phosphate buffer were tested individually, EDTA not, as we already knew from previous experiments that 1 mM EDTA does not cause malformations (unpublished data). Control embryos (n = 20/experiment) were always reared in embryo medium. Positive controls were exposed to rat liver microsomes (200 µg/ml, diluted in embryo medium). The embryos were randomly assigned to a group and the plates were placed in the incubator until further examination.

Table 1. First set of experiments to identify possible toxic components.

Mixture	Sucrose (250 mM)	Phosphate buffer (0.1 M)	EDTA (1 mM)	PI (1 unit/10 ml)	RLM (200 µg/ml)
Mixture 1	**X**				
Mixture 2		**X**			
Mixture 3	**X**	**X**			
Mixture 4	**X**	**X**	**X**		
Mixture 5	**X**	**X**	**X**	**X**	
Mixture 6	**X**	**X**	**X**	**X***	
Mixture 7	**X**	**X**	**X**	**X**	**X**

PI: Halt Protease Inhibitor Single-Use Cocktail, RLM: Rat liver microsomes. *: 1 unit/20 ml.

The second experiment aimed to identify possible additive effects of the protease inhibitor kit and other components of the microsomal mixture. Therefore, we tested the Halt Protease Inhibitor Single-Use Cocktail in absence and presence of other components (see table 2). Each group (controls as well as all test groups) consisted out of 20 embryos. Final exposure concentrations were 0.1 M potassium phosphate buffer, 250 mM sucrose, 1 mM EDTA and 1 unit/ 10 ml of Halt Protease Inhibitor Single-Use Cocktail. Control embryos were reared in embryo medium. The embryos were randomly added to a group and the plates were placed in the incubator until further examination.

Table 2. Second set of experiments to identify the toxicity of PI.

Mixture	Sucrose (250 mM)	Phosphate buffer (0.1 M)	EDTA (1 mM)	PI (1 unit/10 ml)
Mixture 1	**X**			**X**
Mixture 2		**X**		**X**
Mixture 3			**X**	**X**
Mixture 4				**X**

PI: Halt Protease Inhibitor Single-Use Cocktail.

5.2.3.6 Evaluation of rat liver microsomes (RLM) without Halt Protease Inhibitor Single-Use Cocktail

To analyse the toxicity of the RLM-mix without protease inhibitor, we exposed 20 embryos to embryo solution as negative controls (see above), 20 embryos to a preparation of 200 µg/ml RLM without protease inhibitor and 20 embryos to a preparation of 200 µg/ml RLM with 1 unit/10 ml protease inhibitor (positive control). RLM were prepared as described in 2.2.

5.2.3.7 Analysis of co-incubation conditions with non-toxic components

Based upon the results from the previous morphological experiments, a combination of all non-toxic components of the co-incubation system was tested. Controls (n = 20) were reared in embryo medium (see above). Embryos in the test group (n = 20) were exposed to RLM without protease inhibitor (200 µg/ml), with 1.25 mM of NADPH tetrasodium salt as a co-factor. In a second test group (n = 20) RLM without protease inhibitor (200 µg/ml) and 1.25 mM of NADPH tetrasodium salt were co-incubated with 500 µM GA. The last group of embryos (n = 20) were co-incubated with 200 µg/ml HLM, 1.25 mM NADPH tetrasodium salt and 500 µM GA. Mixtures were prepared one hour before exposure to the embryos so that the microsomes were pre-incubated with GA for 1h.

5.2.4 Preparation of rat liver microsomes

One native, not CYP-induced, liver was obtained from a male adult Sprague Dawley rat that was about to be euthanized and served as a control animal in a study approved by the ethical commission for laboratory animal testing of the University of Antwerp (document number: ECD-2014-18). The fresh rat liver tissue was obtained by anaesthetising the rat using 5% isoflurane after which an injection with an overdose of 60 mg/ml sodium pentobarbital in the tail vein was carried out. Afterwards, the entire liver was isolated and flushed with 0.01 M PBS (pH 7.4) until all blood was removed. The entire liver was stored in 0.01 M PBS (pH 7.4) at -80°C until further use.

Liver tissue was manually cut into smaller pieces using scissors and homogenised by means of an electric tissue homogeniser (Kinematica, Lucerne, Switzerland) followed by short ultrasonication. All steps were performed at 4°C in homogenisation buffer consisting of 0.1 M potassium phosphate buffer, 1.15% KCl and 10 mM EDTA dissolved in ultrapure water. One batch of rat liver microsomes (RLM) was prepared with and one without 1 unit/10 ml of Halt Protease Inhibitor Single-Use Cocktail. Subsequently, the homogenised tissue was centrifuged for 10 minutes at 12 000 x g (4°C) and supernatant was transferred to ultracentrifuge tubes. A first ultracentrifugation in ultracentrifugation buffer (0.1 M potassium phosphate buffer, 1.15% KCl and 1 mM EDTA dissolved in ultrapure water) was carried out at 100 000 x g (4°C) for one hour and the pellet was resuspended in ultracentrifugation buffer. Another ultracentrifugation (100 000 x g, 40 minutes at 4°C) was performed and the pellet was resuspended in storage buffer (pH 7.4), which contained 0.1 M potassium phosphate buffer, 250 mM sucrose, 1 mM EDTA and 1 unit/10 ml of Halt Protease Inhibitor Single-Use Cocktail dissolved in ultrapure water. The freshly prepared rat liver microsomes were stored at -80°C until further use.

5.2.5 Reactive oxygen species detection

A tris-HCl buffered medium (work solution of 0.2 M, final concentration of 0.1 M) was used in these experiments because the concentration of the medium in the final mixture (0.1M) is non-toxic for zebrafish embryos and thus relevant for co-incubation studies. The medium was prepared by dissolving 0.588 g/l $CaCl_2.2H_2O$, 0.246 g/l $MgSO_4.7H_2O$, 0.117 g/l $NaHCO_3$ and 0.010 g/l KCl in 0.2 M tris-HCl. ROS was quantified by the fluorescent probe, CM-H_2DCFDA. After reaction of CM-H_2DCFDA with ROS, CM-H_2DCFDA becomes the fluorescent molecule DFCDA. All experiments were replicated four times.

5.2.5.1 ROS detection and the role of the NADPH-regenerating system

The composition of the different premixes is shown in table 3. Human liver microsomes (HLM) were our test samples. Supersomes (SZ) were included as negative controls, as these are insect cell-derived microsomes that lack cytochrome P450 enzymes. After preparing the premixes, they were incubated for 1h at room temperature in a black, flat-bottomed 96-well plate. Afterwards, measurements were initiated by adding the dye (final concentration: 8.67 µM CM-H_2DCFDA), after which the well plate was placed in a Tecan Infinite M200 Pro (Tecan, Männedorf, Switzerland). Fluorescent values were measured every 30 minutes for 2 hours. Excitation and emission wavelengths were 493 nm and 522 nm, respectively.

Table 3. Components of the different premixes and their final concentrations

Mixture	HLM (200 µg/ml)	SZ (200 µg/ml)	NADPH solution A (5%, v/v)	NADPH solution B (1%, v/v)	Medium (0.1 M tris-HCl)
HLM + NRS (Standard)	**x**		**x**	**x**	**x**
HLM	**x**				**x**
SZ + NRS		**x**	**x**	**x**	**x**
SZ		**x**			**x**
NRS			**x**	**x**	**x**

Concentrations mentioned are the final concentrations. HLM: human liver microsomes, NRS: NADPH-regenerating system, SZ: insect cell-derived supersomes (microsomal preparation containing no CYPs)

5.2.5.2 Comparison of ROS-formation of the NADPH-regenerating system and a single dose of NADPH

In order to investigate whether there would be a difference in ROS formation during incubation with an NADPH-regenerating system compared to a single dose of the tetrasodium salt of NADPH, we evaluated ROS formation under these two conditions using the same protocol as in 2.5.1. Mixture C was included as negative control (table 4).

Table 4. Components of the different premixes and their final concentrations

Mixture	HLM (200 µg/ml)	NADPH solution A (5%, v/v)	NADPH solution B (1%, v/v)	NADPH-salt (1.25 mM)	Medium (0.1 M Tris-HCl)
A (Standard)	**x**	**x**	**x**		**x**
B	**x**			**x**	**x**
C		**x**	**x**		**x**

Concentrations mentioned are the final concentrations. HLM: human liver microsomes

5.2.5.3 Characterisation of antioxidant capacity

Gallic acid (GA), rosmarinic acid (RA) and Se-methionine (Se-M), three antioxidants, were tested for their capacity to reduce ROS in the medium. All three antioxidants were added in two concentrations, namely 100 and 500 µM, based upon previous experience in our group with another *in vitro* model[29]. Mixture A is the solution with the standard conditions for co-incubation of zebrafish embryos with HLM and was therefore included as a positive control (table 5). Samples were incubated in the premix for 1h at room temperature in a black, flat-bottomed 96-well plate (Greiner Bio-One, Belgium). After 1h, a final concentration of 8.67 µM CM-H_2DCFDA was added to all wells and the plate was placed in the microplate reader (Tecan, Switzerland). Fluorescent values were measured every 5 minutes for 2 hours with excitation and emission wavelengths of 493 and 522 nm, respectively.

Table 5. Components of the different premixes and their final concentrations

Mixture	HLM (200 µg/ml)	NADPH solution A (5%, v/v)	NADPH solution B (1%, v/v)	GA *	RA *	Se-M *	Medium (0.1 M tris-HCl)
A	**x**	**x**	**x**				**x**
B	**x**	**x**	**x**	**x**			**x**
C	**x**	**x**	**x**		**x**		**x**
D	**x**	**x**	**x**			**x**	**x**
E	**x**			**x**			**x**
F	**x**				**x**		**x**
G	**x**					**x**	**x**

Concentrations mentioned are the final concentrations. * All antioxidants were added in a concentration of 100 µM or 500 µM. Concentrations mentioned are the final concentrations. GA: Gallic acid, HLM: human liver microsomes, RA: Rosmarinic acid, Se-M: Selenium-methionine.

5.2.5.4 Antioxidant capacity of the NADPH-regenerating system (NRS) and a single dose of NADPH

To examine whether there is any difference between the antioxidant capacity of the NRS and a single dose of NADPH tetrasodium salt, we used three antioxidants (GA, RA and Se-M) in a final concentration of 500 µM and incubated each of them with either the NRS or the NADPH-salt. The products were dissolved in 0.1 M tris-HCl buffered medium (see above) and 200 µg/ml of HLM was added to each test condition (table 6). After this, measurements were immediately initiated by placing the plate in the microplate reader (Tecan, Switzerland). Fluorescent values were measured every 5 minutes for 2 hours with excitation and emission wavelengths of 493 and 522 nm, respectively.

Table 6. Components of the different premixes and their final concentrations

Mixture	HLM (200 µg/ml)	NADPH solution A (5%, v/v)	NADPH solution B (1%, v/v)	NADPH-salt (1.25 mM)	Medium (0.1 M Tris-HCl)
NRS + GA	**x**	**x**	**x**		**x**
NRS + RA	**x**	**x**	**x**		**x**
NRS + Se-M	**x**	**x**	**x**		**x**
NADPH + GA	**x**			**x**	**x**
NADPH + RA	**x**			**x**	**x**
NADPH + Se-M	**x**			**x**	**x**

Concentrations mentioned are from the final reaction mixtures. GA: Gallic acid, HLM: human liver microsomes, NADPH: NADPH tetrasodium salt, NRS: NADPH-regenerating system, RA: Rosmarinic acid, Se-M: Selenium-methionine.

5.2.6 CYP activity assay

Ethoxyresorufin is biotransformed into the fluorescent molecule resorufin by CYP1A (*i.e.* ethoxyresorufin-O-deethylation or EROD). As such, the amount of resorufin formed can be used as an indication of CYP activity. All experiments consisted out of 4 technical replicates.

5.2.6.1 Comparison of CYP activity of the NADPH-regenerating system and a single-dose of NADPH-salt

Because NADPH tetrasodium salt was not embryotoxic (see section 3.3), we tested whether CYP activity was similar as in NRS. Samples were prepared by adding 200 µg/ml of HLM to 0.1 M tris-HCl buffered medium. The tris-HCl buffered medium consisted of 0.294 g/l $CaCl_2$. $2H_2O$, 0.123 g/l $MgSO_4$. $7H_2O$, 0.059 g/l $NaHCO_3$ and 0.005 g/l KCl dissolved in 0.1 M tris-HCl. Next, either 1.25 mM of NADPH tetrasodium salt or, respectively, 5 and 1% of NADPH solution A and B was added to the medium. This was followed by adding 10 µM ethoxyresorufin to all samples and 200 µl of all samples was loaded in a black flat-bottomed 96 well plate (Greiner Bio-One, Belgium). Immediately afterwards, the plate was

placed in a microplate reader (Tecan, Switzerland) and measurements were initiated. Every 5 minutes for 2 hours, fluorescence was measured with excitation and emission wavelengths of 550 and 590 nm, respectively.

5.2.6.2 Evaluation of RLM without Halt Protease Inhibitor Single-Use Cocktail

For the preparation of rat liver microsomes, see section 2.2. Samples were prepared by adding either 5 or 10 µg/ml RLM which were prepared with or without 1 unit/10 ml Halt Protease Inhibitor Single-Use Cocktail (see 2.2). Next, we added 0.1 M potassium phosphate to 0.1M tris-HCl buffered medium (0.294 g/l $CaCl_2.2H_2O$, 0.123 g/l $MgSO_4.7H_2O$, 0.059 g/l $NaHCO_3$ and 0.005 g/l KCl dissolved in 0.1 M tris-HCl). NADPH solution A and B were added as 5 and 1% of the reaction mixture, respectively. Afterwards, 10 µM of ethoxyresorufin was added to all samples and the same protocol as in 2.6.1 was used.

5.2.6.3 Analysis of co-incubation conditions with non-toxic components

To test the activity of protease inhibitor free RLM in combination with all other non-toxic co-factors, 200 µg/ml of the RLM were dissolved in 0.1 M tris-HCl buffered medium with 0.294 g/l $CaCl_2.2H_2O$, 0.123 g/l $MgSO_4.7H_2O$, 0.059 g/l $NaHCO_3$ and 0.005 g/l KCl. Next, either 1.25 µM of NADPH tetrasodium salt or, respectively, 5 and 1% of NADPH solution A and B was added to the medium. Both mixtures were tested as such, but also with 500 µM of GA added to them. Reactions were initiated by adding the product (10 µM ethoxyresorufin) to all samples and the same protocol as In 2.6.1 was used.

5.2.7 Statistics

All statistic results were considered significant if the p-values (corrected for multiple testing, where applicable) were ≤ 0.05. For the fluorescent reactive oxygen species assay, we analysed the amount of ROS formed after 120 minutes by means of the non-parametric Kruskal-Wallis test with correction for multiple testing (Dunn's test). If only two groups had to be compared separately, a Mann Witney-U test was used. For the CYP activity assays, we analysed the velocity of resorufin formation in the linear part of the curve using the non-parametric Kruskal-Wallis test with correction for multiple testing (Dunn's test). If only two groups had to be compared, a Mann Witney-U test was used.

Morphological data could not be analysed using the chi-squared test, as the expected frequencies of many cells of the contingency Tables were too low (≤ 5). Therefore, we analysed these data using the non-parametric Fisher's exact test. All statistical tests were performed using Graphpad Prism 7 (San Diego, CA, USA).

5.3 Results

5.3.1 Morphological comparison of the effects after short- and long-term exposure

The results of dimethadione are represented in table 7. Exposure to dimethadione for 1h did not result in significant effects at any of the concentrations tested compared to controls ($p \geq 0.0661$). After 70h of exposure, there was a clear effect at the highest concentration of dimethadione (30 mM) in terms of lethality and malformations ($p < 0.0001$), as 100% of the embryos were affected. No significant effects were noted at the other concentrations. Both the MAS control group and the MAS test group with trimethadione did not show an increase in coagulation or malformations ($p \geq 0.0648$) compared to controls. Long-term (70h) exposure to 30 mM trimethadione showed an increase in malformations compared to the controls ($p \leq 0.0022$), which affected 45% of the embryos and thus approximating the EC_{50} for this compound.

Table 7. Summary of the lethal and teratogenic effects of dimethadione after short- and long-term exposure.

	Normally developed embryos (%)		Embryos with teratogenic effects (%)		Embryos with lethal effects (%)		Affected embryos (%)	
	1h exposure	70 h exposure	1h exposure	70 h exposure	1h exposure	70 h exposure	1h exposure	70 h exposure
Controls		92.5 ± 2.5		5 ± 5		2.5 ± 2.5		7.5 ± 2.5
0.03 mM DM	85 ± 0	85 ± 2.5	7.5 ± 2.5	5 ± 5	7.5 ± 2.5	10 ± 0	15 ± 0	15 ± 5
0.3 mM DM	75 ± 0	87.5 ± 7.5	12.5 ± 7.5	12.5 ± 7.5	12.5 ± 7.5	0 ± 0	25 ± 0	12.5 ± 7.5
3 mM DM	80 ± 5	77.5 ± 7.5	5 ± 5	12.5 ± 7.5	15 ± 0	10 ± 0	20 ± 5	22.5 ± 7.5
30 mM DM	77,5 ± 2.5	0 ± 0	12.5 ± 7.5	52.5 ± 12.5	10 ± 10	47.5 ± 12.5	22.5 ± 2.5	100 ± 0

Controls were reared in embryo medium for the entire duration of the experiment. Significant differences compared to the controls are marked in grey. DM: dimethadione

5.3.2 Embryotoxicity of the supernatant of MAS

Already at 24 hpf (figure 3), several embryos in the group exposed to the supernatant of ultracentrifuged human liver microsomes died ($p < 0.001$). After 48 hpf, all embryos in the supernatant group died whilst the controls had a survival of 97.5% ($p < 0.0001$). At the end of the experiment (96 hpf), survival of the controls was still above 95%.

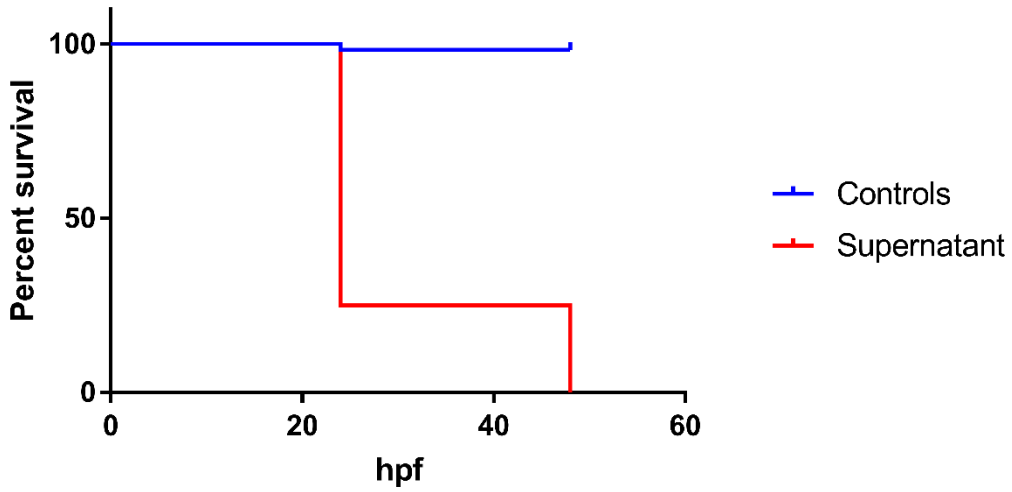

Figure 3. Survival curve of the embryos exposed to embryo medium (controls) and the embryos exposed to the supernatant of human liver microsomes after 1h pre-incubation and subsequent ultracentrifugation.

5.3.3 Reactive oxygen species in the mDarT

5.3.3.1 ROS detection and the role of the NADPH-regenerating system (NRS)

High levels of ROS were present in three groups (HLM + NRS, HLM and SZ, p-values ≤ 0.0286) compared to the negative control (NRS). SZ + NRS showed no significant ROS concentration (p-value = 0.0571) (figure 4). There was no difference in ROS between HLM + NRS and HLM ($p = 0.6857$), whereas SZ showed a higher ROS concentration compared to SZ + NRS ($p = 0.0286$).

5.3.3.2 Comparison of ROS-formation of the NADPH-regenerating system (NRS) and a single dose of NADPH

There was no difference in ROS formation between HLM + NRS and HLM + NADPH (p-value = 0.1000). The negative control (NRS) gave no signal.

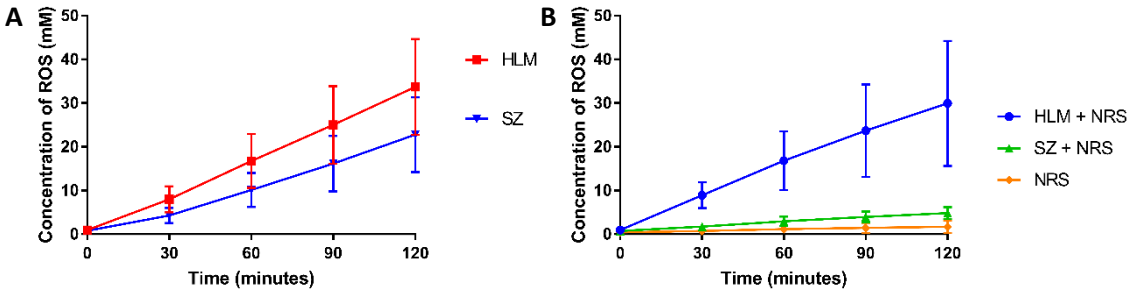

Figure 4. ROS formation over time of human liver microsomes and supersomes up to 2h incubation**. A.** HLM: Mixture with only human liver microsomes, SZ: Mixture with only supersomes**, B.** HLM + NRS: Mixture with human liver microsomes and the NADPH-regenerating system, SZ + NRS: Mixture with supersomes and the NADPH-regenerating system, NRS: Mixture with only the NADPH-regenerating system.

5.3.3.3 Characterisation of antioxidant capacity

The amount of ROS of the standard MAS condition (HLM + NRS) was compared to all three antioxidants in the highest concentration. For 500 µM of selenium-methionine there was no difference in ROS levels compared to the standard (p-value = 0.0571) while there was a difference between 500 µM gallic acid and the standard and 500 µM rosmarinic acid and the standard (p-value ≤ 0.0286). We then compared whether there was a difference in ROS reduction between the high and low concentration of all antioxidants. This was not the case as p-values ≥ 0.0571 for all three antioxidants (figure 5).

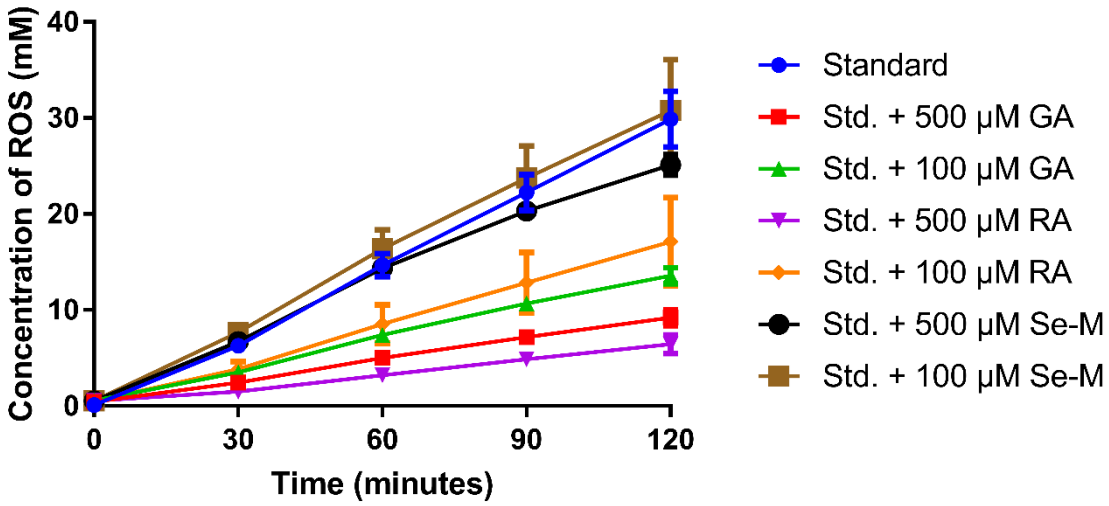

Figure 5. Effects of three antioxidants on microsomal ROS in both a high and low concentration. Standard (Std.) includes human liver microsomes and the NADPH-regenerating system. GA: Gallic acid, RA: Rosmarinic acid, Se-M: Selenium-methionine.

5.3.3.4 Antioxidant capacity in presence of the NADPH-regenerating system and a single dose of NADPH salt

For every antioxidant, ROS formation of the NRS was compared to a single dose of NADPH-salt. NRS + gallic acid, rosmarinic acid or selenium-methionine did not show any differences in ROS concentration to their NADPH-salt counterparts with p-values ≥ 0.2000 (figure 6).

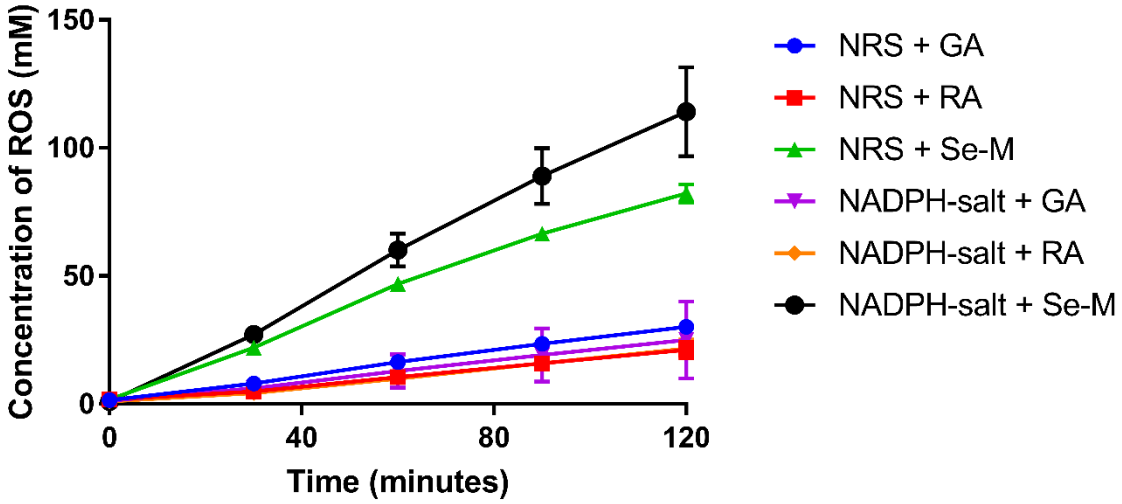

Figure 6. Comparison of antioxidant capacity of NRS and a single dose of the NADPH-salt. The curves of NRS+RA and NADPH-salt + RA overlap almost entirely. GA: Gallic acid, NRS: NADPH-regenerating system, RA: Rosmarinic acid, Se-M: Selenium methionine.

5.3.3.5 Morphological effects of gallic acid on zebrafish embryos during co-incubation with HLM

As gallic acid showed a pronounced reduction in MAS-induced free radicals in the medium and was very soluble in water, this antioxidant was further used in the morphological experiments. Table 8 summarises the morphological outcome at 96 hpf for different incubation conditions with and without gallic acid. The embryos in the negative control group (embryo medium) developed normally. The embryos co-incubated with HLM and the NADPH-regenerating system (positive control) all died at 24 hpf. The gallic acid controls (*i.e.* the embryos reared in embryo medium with only 100 or 500 µM of gallic acid) showed no malformations, thus gallic acid is non-toxic during zebrafish organogenesis. However, it did not reduce the embryotoxicity of the MAS, as zebrafish embryos co-incubated with human liver microsomes, NADPH-regenerating system and gallic acid (both 100 µM and 500 µM) all died after 24 hpf. Consequently, further examination of these groups and the positive controls was not possible as no embryo survived beyond 48 hpf. Embryos in both the medium control group and gallic acid control groups developed normally throughout the rest of organogenesis (*i.e.* 96 hpf, data not shown).

Table 8. Summary of malformations at the end of organogenesis (96 hpf).

Group	Coagulation	Oedema Pericard	Blood accumulation yolk
Negative control (medium)	0/12	1/12	0/12
Positive controls (HLM + NRS)	12/12 p < 0.0001	CNE	CNE
100 µM GA control	0/12 p >0.9999	0/12 p >0.9999	0/12 p >0.9999
500 µM GA control	0/12 p >0.9999	1/12 p >0.9999	1/12 p >0.9999
100 µM GA co-incubation with HLM	12/12 p < 0.0001	CNE	CNE
500 µM GA co-incubation with HLM	12/12 p < 0.0001	CNE	CNE

P-values compared to the control group. Significant p-values are marked in grey CNE: Could not be examined (due to no surviving embryos in that group).

5.3.4 Examination of toxic components in the mDarT

5.3.4.1 Morphological evaluation of the toxicity of the different components of a microsomal mixture

Controls of both experiments of this set showed less than 10% coagulation and no significant differences amongst different experiments (data not shown). Therefore, we pooled the control embryos for further statistical analysis.

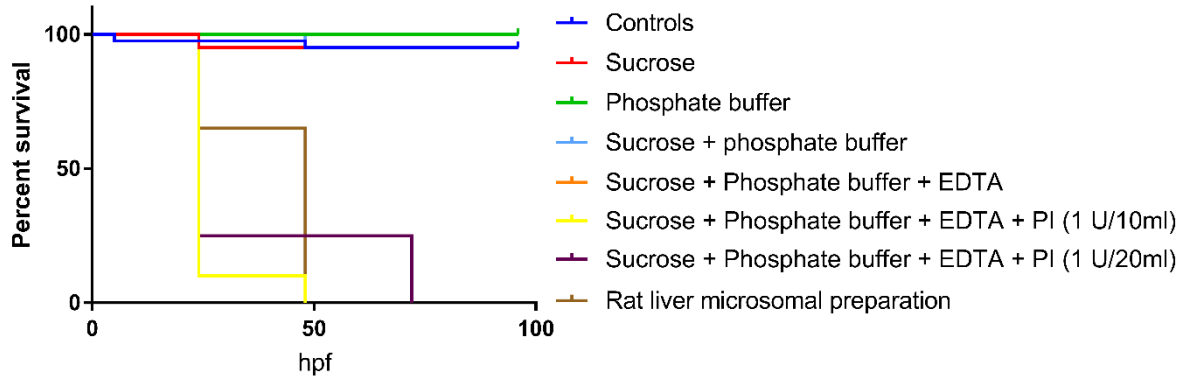

Figure 7. Survival curve of the controls and all 7 mixtures tested. From 24 hpf onward, mixtures including protease inhibitor cocktail (bottom three) had statistically lower percentages of surviving embryos. The curves of Sucrose + phosphate buffer and Sucrose + phosphate buffer + EDTA overlap with the curve of Phosphate buffer until 48 hpf, after which they overlap with the controls and Sucrose.

100% mortality was observed at 96 hpf in the 3 last three groups (see figure 7). All three of these groups had the Protease Inhibitor Single-Use Cocktail (PI) in it ($p < 0.0001$ compared to controls). Therefore, we also statistically examined the earlier time points starting with 72 hpf and going back to 5 hpf (see Figure 6). For all 3 mixtures, coagulation was 100% at 72 hpf as well ($p < 0.0001$). At 48 hpf, coagulation appeared to be 100% for Sucrose + phosphate buffer + EDTA + PI (1 U/10ml) and Rat liver microsomal preparation ($p < 0.0001$). For the mixture with diluted PI (1 U/20ml), only 5 out of 20 embryos survived, which was still statistically lower than the controls ($p < 0.0001$). Moreover, the surviving embryos had a bigger chance of developing blood accumulations in the tail and deviations of the ear (p-values < 0.0001). At 24 hpf, there was also already an increase in mortality in the mixtures with PI in them compared with the controls, with p-values ≤ 0.0132. For Sucrose + phosphate buffer + EDTA + PI (1 U/10ml) and Rat liver microsomal preparation, there was an increased amount of embryos that had indistinguishable body parts ($p \leq 0.0012$). The embryos exposed to Sucrose + phosphate buffer + EDTA + PI (1 U/20ml) died later in development compared to its equivalent with 1 unit/10 ml of Halt Protease Inhibitor Single-Use Cocktail (p-values for 24 hpf and 48 hpf were ≤ 0.0471).

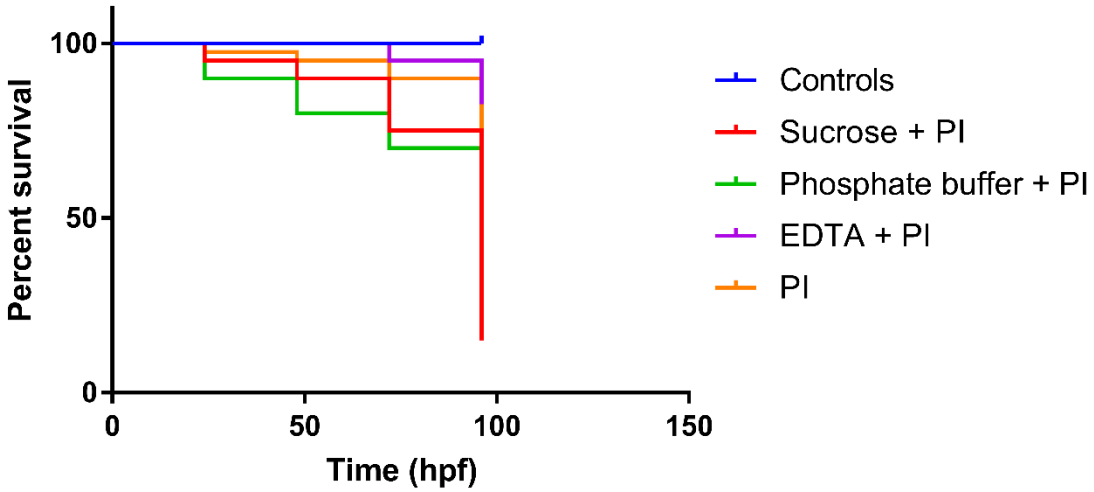

Figure 8. Survival curve of the controls and all components with protease inhibitor cocktail. From 48 hpf onward, Phosphate buffer + PI had statistically lower percentages of surviving embryos ($p = 0.0053$). Sucrose + PI ($p = 0.0010$) showed decreased survival after 72 hpf and the amount of coagulated embryos in the EDTA-containing mixture ($p = 0.0117$) was only significantly higher at the end of organogenesis (96 hpf).

In the second experiment with protease inhibitor cocktail, all groups had an increased amount of coagulated embryos ($p \leq 0.0117$) at the end of organogenesis compared to the controls (see figure 8). However, in contrast with some groups of experiment 1, 100% lethality was not present during these experiments. The mixture containing phosphate buffer + protease inhibitor cocktail and the mixture with only protease inhibitor cocktail did not differ significantly in terms of coagulated embryos ($p = 0.0685$). This was also the case for EDTA + protease inhibitor cocktail and only protease inhibitor

cocktail ($p = 0.2933$). However, Phosphate buffer + protease inhibitor cocktail showed an increased amount of coagulated embryos compared to EDTA + PI ($p = 0.0020$). All other comparisons were also significantly different, with p-values ≤ 0.0033.

At 96 hpf, several malformations had an increased incidence in some of the test groups compared to the controls. These parameters can be found in table 9 with their incidences and respective p-values.

Table 9. Overview of coagulation and malformations increased in at least one test group at 96 hpf

Mixture	Coagulation	Failure to hatch	Curved tail	Oedema pericard	Malformation yolk	No blood circulation in tail	Disturbed blood circulation in tail	Deviating shape of head	Deviating shape of mouth	Deviating pigmentation
Controls	0/40	0/40	1/40	0/40	0/40	0/40	1/40	0/40	0/40	0/40
Sucrose + PI	34/40 p < 0.0001	1/6 p = 0.1304	0/6 p > 0.9999	3/6 p = 0.0013	4/6 p < 0.0001	4/6 p < 0.0001	0/6 p > 0.9999	4/6 p < 0.0001	4/6 p < 0.0001	2/6 p = 0.0145
Phosphate buffer + PI	21/40 p < 0.0001	0/19 p > 0.9999	4/19 p = 0.0333	6/19 p = 0.0006	4/19 p = 0.0085	1/19 p = 0.3220	6/19 p = 0.0033	1/19 p = 0.3220	1/19 p = 0.3220	0/19 p > 0.9999
EDTA + PI	7/40 p = 0.0117	0/33 p > 0.9999	1/33 p > 0.9999	9/33 p = 0.0004	6/33 p = 0.0065	4/33 p = 0.0376	7/33 p > 0.0195	3/33 p > 0.0877	3/33 p > 0.0877	1/33 p > 0.9999
PI	12/40 p = 0.0002	20/28 p = 0.0004	0/28 p > 0.9999	13/28 p < 0.0001	11/28 p < 0.0001	5/28 p = 0.0094	9/28 p = 0.0010	5/28 p > 0.0094	4/28 p = 0.0251	0/28 p > 0.9999

Statistically significant ($p \leq 0.05$) values are marked in grey, all transparent cells were not statistically different from the controls as indicated by their p-values.

5.3.4.2 Evaluation of CYP activity and embryotoxicity by RLM without Halt Protease Inhibitor Single-Use Cocktail

The velocity of resorufin formation was higher at 10 µg/ml rat liver microsomes without protease inhibitor cocktail than at 5 µg/ml of rat liver microsomes both with or without protease inhibitor cocktail in the mixture (p-values ≤ 0.0227) (figure 9). For all other pairwise comparisons, the velocity did not differ, with p-values ≥ 0.3803.

However, omission of protease inhibitor cocktail did not reduce embryotoxicity. At the earliest time points, we already observed abnormalities in the test group (RLM without PI) and the positive control group (RLM with PI). After 24 hours, all embryos in both of these groups died, while the negative controls only had 1 embryo out of 20 that died ($p < 0.0001$). Controls developed normally throughout organogenesis and the amount of malformed embryos was lower than 5%.

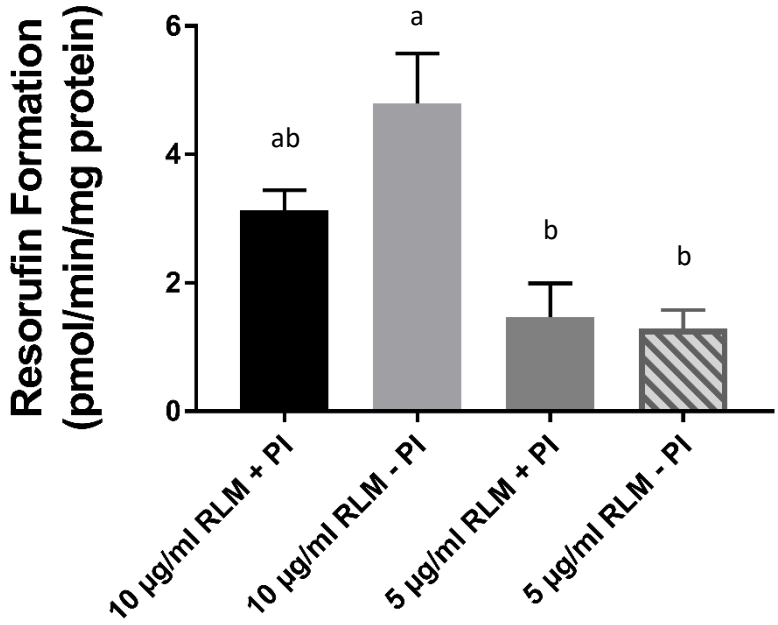

Figure 9. Velocity of microsomal resorufin formation of two doses of rat liver microsomes (RLM) prepared with or without protease inhibitor cocktail (PI). Statistical differences between groups are indicated by different letters above the bar chart.

5.3.5 Evaluation of CYP activity and embryotoxicity of a single dose of NADPH tetrasodium salt

The results for CYP activity are shown in Figure 10. The two groups were not statistically different, with Tris + NRS having the same velocity of resorufin formation compared to single dosing of NADPH (p-value = 0.2863).

Regarding embryotoxicity, we noted earlier that NADPH-regenerating system caused toxicity when incubated with zebrafish embryos, leading to lethality after 24-48 hpf (data not shown). Because the NRS consists of multiple components, we tested whether NADPH tetrasodium salt was also embryotoxic. NADPH tetrasodium salt did not cause any malformations or coagulation (all p-values ≥ 0.4305).

5.3.6 Analysis of CYP activity and embryotoxicity in co-incubation conditions with non-toxic components

We compared NRS and NADPH-salt without gallic acid with their counterpart with gallic acid added to it (figure 10). For rat liver microsomes incubated with the NADPH-regenerating system, the amount of resorufin formation was higher than when gallic acid was added to the mix (p-value = 0.0286). This was also the case for rat liver microsomes + NADPH-salt, which signal was higher than when gallic acid was included (p-value = 0.0286).

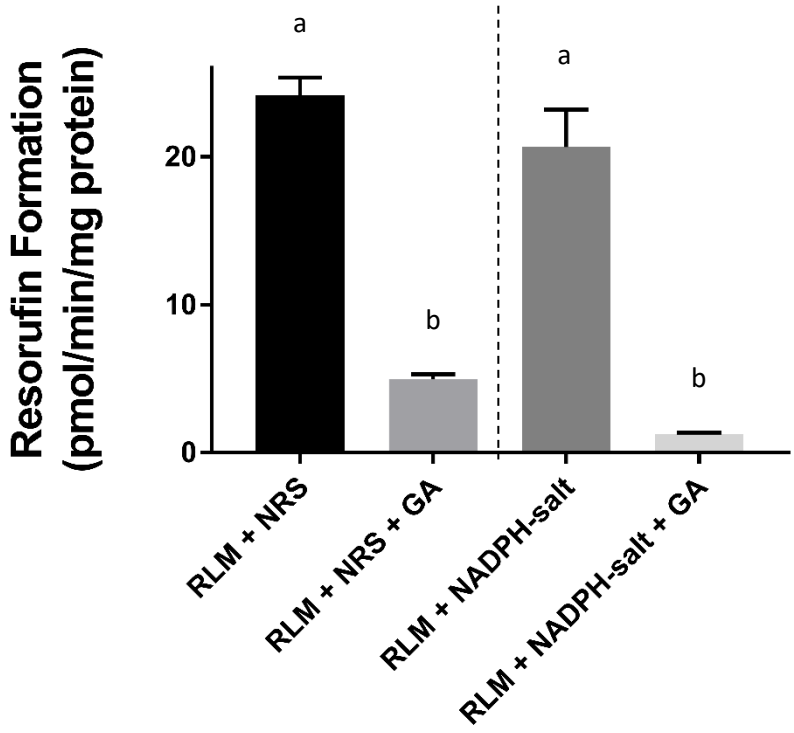

Figure 10. Velocity of microsomal resorufin formation of rat liver microsomes with the co-factor either being the NADPH-regenerating system or a single dose of NADPH-salt in presence and absence of gallic acid. GA: gallic acid. Statistical differences for the NADPH-regenerating system and NADPH-salt groups are indicated by different letters above the bar chart.

Embryos exposed to rat liver microsomes without protease inhibitor and the NADPH-salt as co-factor showed signs of toxicity. Pre-incubation with 500 µM gallic acid did not reduce embryotoxicity. After 5 hpf and 10 hpf (figure 11), embryos were not yet coagulated but the cells appeared to be loosened from the yolk compared to the controls. At 24 hpf, all embryos in the test groups died ($p < 0.0001$), whilst no control embryos showed any apparent signs of malformations. At the end of organogenesis, only one control embryo died (around 72 hpf) and the amount of malformed embryos was lower than 5%.

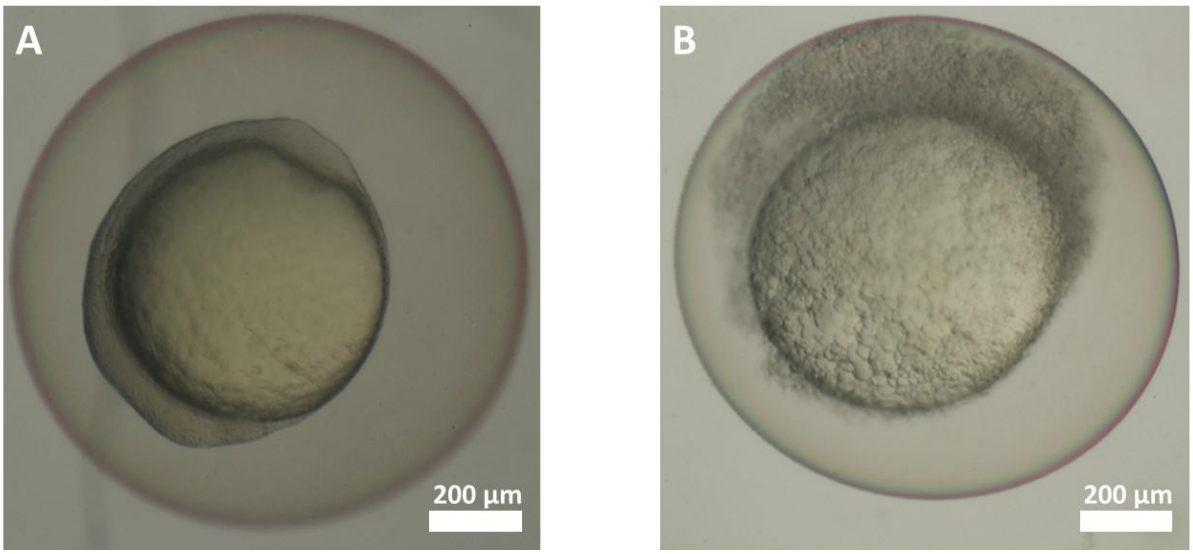

Figure 11. Embryos at approx. 8 hpf. A: embryo developing normally, showing no signs of abnormalities. B: No epiboly took place with loose cells on top of the yolk as a result. Embryo was scored as coagulated at 24 hpf.

5.4 Discussion

In this study, we first assessed whether short co-incubation with MAS in the mDarT and consequently short exposure to the parent compound and its metabolites for 1h is a risk to miss potential teratogens. As Weigt et al[8] suggested that the observed teratogenic effect of trimethadione (TM) was caused by its (re)active metabolite dimethadione (DM), we exposed zebrafish embryos to several concentrations of DM for either 1h or during organogenesis. We also included TM at the estimated EC_{50}[8], as a positive control, and indeed about 45% of zebrafish embryos were affected after exposure from 2 until 72 hpf. However, our study showed that 1h of co-incubation of zebrafish embryos with TM and MAS was insufficient because no significant effects were observed at 72 hpf compared with controls. This was in line with our data of its metabolite DM after 1h of exposure at different concentrations. Even at the highest concentration, 30 mM, no significant effects were noted. This is in contrast to findings with acetaminophen, for which a clear concentration-relationship in effects was observed after co-incubation with MAS for 1h[8]. This confirms again that the onset of teratogenic action may differ

between compounds. Indeed, when exposing zebrafish embryos longer to DM, i.e. from 2 until 72 hpf, a clear effect was noted at the highest concentration, *i.e.* 30 mM, at which all embryos were affected. Also more pronounced effects were noted for DM compared with TM at the same concentration. So, although it appears that besides the active metabolite DM also the parent compound TM itself may contribute to the observed developmental defects observed after long-term exposure, our study clearly showed for TM that 1h co-incubation with MAS was not sufficient to elicit an effect in zebrafish embryos and that a longer exposure window was required.

Considering the above findings, we investigated whether modifications in the mDarT protocol could reduce embryotoxicity of MAS and as such lead to a larger exposure window. Given the fact that omission of the microsomes from the incubation medium after pre-incubation for 1h did not resolve or even reduce embryotoxicity and oxidative drug metabolism might cause increased levels of reactive oxygen species (ROS) in the medium in which NADPH can play a pivotal role, we investigated the effects of ROS on zebrafish embryonic development in the mDarT.

In our study, we found that microsomes produce reactive oxygen species. However, this cannot solely be addressed to the CYP activity in the liver microsomes, as we also detected ROS in a mixture of microsomes without the necessary co-factor NADPH for CYP activity and in supersomes containing no CYPs. Thus microsomes are capable of producing free radicals without CYP activity. This is probably due to other (enzymatic) processes for which no NADPH is required. Interestingly, microsomes incubated with NRS even had a lower ROS concentration compared to microsomes in absence of NRS which indicates that NADPH can, in fact, reduce the amount of radicals via the microsomal glutathione S-transferases[22]. These results were also confirmed by the mixtures with supersomes, in which the mixture with NRS had a ROS signal that was negligible, whilst the supersomes group without NRS showed high levels of free radicals.

Addition of gallic and rosmarinic acids successfully decreased the amount of ROS, whereas Se-methionine failed to reduce ROS levels even at a high concentration (500 µM). Subsequently, one antioxidant (GA) was used for further morphological testing. Previously, it has been described that a single injection of GA caused several teratogenic effects in developing chicken embryos such as stillbirth and reduced body weight[30,31]. In our study, we preferred continuous exposure to GA to cover the entire period of organogenesis. GA did not cause any detrimental effects on zebrafish development, not even at the highest concentration when compared to control embryos reared in embryo medium. Therefore, it can be concluded that GA is not teratogenic for zebrafish embryos at the concentrations used. When we co-incubated zebrafish embryos with human liver microsomes and NRS in the presence of a high and low dose of GA, the co-incubation with GA could not prevent the 100% lethality that occurred after 24 hpf. In conclusion, we were able to detect microsomal ROS

formation, which is not caused by CYP activity exclusively. Microsomal ROS can be reduced by antioxidants in the medium and the selected antioxidant, GA, was non-toxic for zebrafish during organogenesis. However, GA was incapable of reducing the toxicity of MAS which means that free radicals are present, but not the only reason for the observed embryotoxicity during co-incubation with mammalian liver microsomes.

As ROS is not the main cause of embryotoxicity in the mDarT, we also investigated whether components of the microsomal mixture contributed to the toxicity during co-incubation. Protease inhibitor caused 100% lethality in every group where it was added. This is not surprising, as the protease inhibitor cocktail (PI) consists of several components that were already described as being embryotoxic[32,33]. An example is the inhibitor aprotinin, which has been described to cause lethality between the gastrula and neurula stage in Xenopus embryos[32]. Moreover, some other components of this protease inhibitor cocktail also cause non-lethal developmental effects, such as leupeptin which caused internal and external abnormalities in rats[33]. Whether these components of the protease inhibitor cocktail also exert effects on zebrafish embryos was beyond the scope of this article. However, the tested cocktail proved to be toxic and the toxicity was dose dependent since a concentration of 1 unit/20 ml showed lower lethality rates compared to embryos exposed to 1 unit/10 ml. However, interactions of the PI with other components could also play a role, as not all mixtures exerted the same effects at the same developmental stage. PI together with sucrose for example caused a death rate of 85% at the end of organogenesis, while EDTA had the lowest amount of coagulated embryos (17.5%). Mixtures without PI showed no increase of coagulation or toxicity, making this seemingly the critical factor for MAS toxicity. Surprisingly, co-incubation of zebrafish embryos with in-house prepared RLM without PI also showed lethal effects. This indicates that the microsomes themselves are causing toxicity in the mDarT.

Because NRS is embryotoxic and consists out of several products (*i.e.* Glucose-6-phosphate, Glucose-6-phosphatedehydrogenase, $MgCl_2$, $NADP^+$ and sodium citrate), we also tested whether toxicity could be reduced by replacing NRS by a single dose of NADPH tetrasodium salt. The embryos exposed to the salt did not develop any malformations and did not die. Therefore, we continued testing the NADPH-salt to see if it had an influence on CYP activity. By means of an EROD assay, we noted that CYP activity was comparable for the NADPH-salt and the NRS. Therefore, the rate-limiting step in the reaction is the enzymatic activity, rather than the availability of NADPH. The oxidative stress produced by the salt was in the same magnitude as for the NRS. Because the NADPH-salt did not trigger toxicity and showed CYP activity, it is advised to use the NADPH tetrasodium salt in the mDarT and no longer use an NRS.

The final experiment combined all non-toxic components (*i.e.* in-house prepared rat liver microsomes without protease inhibitor cocktail, with NADPH tetrasodium salt and gallic acid dissolved in a non-toxic embryo medium) but yet lethality occurred after 24 hpf.

5.5 Conclusion

Co-incubation of zebrafish embryos with MAS causes an increase in ROS in the medium of the mDarT. The ROS-levels can be reduced by administering an antioxidant such as gallic acid, but embryotoxicity in the mDarT is still present. Using a single dose of NADPH or modifications of the MAS preparation did also not reduce embryotoxicity in the mDarT. As such, the endoplasmic reticulum appears to possess inherent embryotoxic properties that are not linked to CYP-activity. Therefore, the exposure window in the mDarT could not be extended by reducing ROS levels, single dosing of NADPH or modifications of the MAS preparation.

5.6 Acknowledgements

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Chapter 6: A pre-incubation strategy for metabolization in the metabolic zebrafish developmental toxicity assay (mZEDTA)

Abstract

The zebrafish embryo has gained interest as an alternative model for developmental toxicity screening of drugs the last few decades. However, the lack of biotransformation of compounds by the embryo itself during the major part of organogenesis remains an issue. In order to resolve this drawback, several research groups have investigated the application of an exogenous metabolic activation system (MAS) for coincubation purposes with zebrafish embryos. However, the current MAS protocols using human or rat liver microsomes are toxic for zebrafish embryos hampering coincubation during the entire period of organogenesis. We previously evaluated the effects of temperature, the composition of the medium and ROS in the coincubation medium and saw that some are important factors contributing to the observed toxicity of MAS. However, even when these factors were optimized, embryotoxicity of the system was observed when the microsomes were present. Therefore, we opted for a new approach in the present study, i.e. preincubation of the compound with MAS followed by dilution prior to exposure to the zebrafish embryos. However, a 1/20 dilution was required to omit embryotoxicity of MAS. As a result, the concentrations of the metabolites may be too low. Therefore, an additional step after preincubation was added, *i.e.* the preincubate was ultracentrifuged and the supernatant was diluted. By means of this approach, a dilution factor of 1/10 was sufficient to avoid embryotoxic effects. This protocol was finally validated with a tool compound, trimethadione. The positive control (which was the undiluted preincubation mixture) caused 100% lethality. The group exposed to 30 mM trimethadione without a MAS showed several malformations which aggravated with exposure length. However, the groups exposed to the presumptive teratogenic metabolite did not cause pronounced developmental toxicity. This hints towards a teratogenic, rather than a proteratogenic effect for trimethadione. This was in line with results of a 1/10 diluted preincubation exposure, for which the embryos did not suffer from any developmental toxicity, but this should be confirmed with LC-MS analysis of the internal concentration.

6.1 Introduction

Previous studies using zebrafish embryos for developmental toxicity screening show that the predictivity of the zebrafish embryofoetal development toxicity assay (ZEDTA) is high for known mammalian teratogens but can still be improved[1]. Besides a lack of harmonised protocols, one of the limitations of the ZEDTA is the lack of xenobiotic biotransformation during a major part of organogenesis[2,3]. As zebrafish eggs are fertilised externally, no maternal metabolism is present in the ZEDTA, in contrast to mammals, in which compounds undergo first pass metabolism in the liver of the mother before reaching the foetus. Proteratogens, which are only causing developmental effects after bioactivation, can therefore be missed in the ZEDTA. As a result, several research groups, including

ours, have been exploring the use of an exogenous Metabolism Activation System (MAS) as a coincubation system for the ZEDTA, also called metabolic Danio rerio Test (mDarT) by some groups[4–7]. The first group that introduced a MAS was Busquet *et al*. in 2008[4]. Interestingly, they could only coincubate the MAS with zebrafish embryos for 1 to 2 hours as the coincubation conditions were embryotoxic themselves. However, for developmental toxicity testing exposure to the test compound and its metabolites should be guaranteed during the entire period of organogenesis. Thus, exposing the zebrafish embryos for such a short period may be insufficient as the critical window for a certain teratogen has been missed. Other groups reported this issue as well and Mattsson *et al*. (2012) were able to extend the exposure window up to 4 hours depending on the developmental stage[6]. As organogenesis in zebrafish embryos is completed in approximately 90 hours after the start of gastrulation, 4 hours of exposure is still far from ideal.

Therefore we aimed to extend the exposure window of zebrafish embryos to parent compounds and their human metabolites during the entire period of organogenesis. In previous studies, we noted however that co-incubation of zebrafish embryos with human liver microsomes for longer than 1h at the start of gastrulation resulted in embryotoxicity, similar to a report by Busquet *et al.* (2008)[4]. Alterations of the coincubation conditions and preincubation with antioxidants proved to be unsuccessful (data submitted). From these studies it appeared that liver microsomes (human, but also zebrafish) possess an intrinsic capability to cause embryotoxicity. Therefore, coincubation of zebrafish embryos with human liver microsomes during organogenesis is not possible. The aim of this study was to investigate whether new approaches in the form of preincubation of the MAS followed by coincubation with zebrafish embryos could resolve embryotoxicity. Therefore, we first reared zebrafish embryos in three dilutions (1/10, 1/20 and 1/30, with respective concentrations of 20, 10 and 6.67 µg proteins/ml) of the MAS (200 µg proteins/ml) with zebrafish embryos. Next we included an ultracentrifugation step prior to dilution of the samples to see whether separation of the supernatant with the compound and metabolites from the pellet, containing the microsomes, might resolve embryotoxicity issues.

6.2. Material and methods

6.2.1 Chemicals and products

NADPH tetrasodium salt was purchased from Roche (Vilvoorde, Belgium), Calcium chloride ($CaCl_2.2H_2O$) and ethyl 3-aminobenzoate methanesulfonate (MS-222) were purchased from Sigma-Aldrich (Diegem, Belgium). CM-H_2DCFDA, tris-HCl (1M, pH 7.5) and ultrapure water were purchased from Thermo Fisher Scientific (MA, USA). Potassium chloride (KCl) was purchased from Merck (Overijse, Belgium), sodium bicarbonate ($NaHCO_3$) from Acros Organics (Geel, Belgium) and magnesium sulphate ($MgSO_4.7H_2O$) from UCB Pharma (Leuven, Belgium). Trimethadione was purchased from Tebu-Bio (Boechout, Belgium). DMSO was purchased from Fischer Scientific (Leicestershire, UK). The human liver microsomes (pooled, 50 donors, lot PL050 B) were purchased from Gibco (Thermo Fischer scientific, USA). Instant Ocean Sea Salt was purchased from Instant Ocean (Blacksburg, VA, USA).

6.2.2 Adult zebrafish housing and egg collection

An adult zebrafish breeding stock (*Danio rerio*, in house wild type AB zebrafish line) was maintained. The fish were kept in tanks of approximately 50 liters. The water temperature was set to 28.5 ± 0.2°C and the conductivity and pH of the water were 500 ± 25 µS/cm and 7.5 ± 0.3 by means of Instant Ocean sea salts and $NaHCO_3$, respectively. Water renewal was carried out when ammonia, nitrite and nitrate reached detectable levels. An automated light cycle was being used in the room with a 14/10h light/dark cycle. The fish were fed daily thawed food (*Artemia nauplii*, *Daphnia*, *Chironomidae* larvae or *Chaoborus* larvae), as well as granulated food (Biogran medium; Prodac international, Cittadella, Italy) at a rate of 2% of their mean wet weight per feeding. The Ethical Committee for Animal Experimentation from University of Antwerp (Belgium) approved the use of the animals (ECD 2015-51).

The day before mating, all fish were transferred to a net in the tank to prevent the adults from eating the eggs. The next morning, the fish were allowed to spawn eggs and fertilise them for a period of approximately 45 minutes, after which the embryos were collected from the bottom of the tank by siphoning them out with a tube. To remove faeces and coagulated eggs, multiple washing steps in tris-buffered embryo medium, *i.e*. 0.294 g $CaCl_2.H_2O$, 0.123 g $MgSO_4.7H_2O$, 0.059 g $NaHCO_3$, 0.005 g KCl and 0.1M tris-HCl (pH 7.5), dissolved in 1 litre reverse osmosis water, were performed. Within 2 hours post fertilisation (hpf), embryos were checked under a Leica S8 APO microscope with Leica L2 lighting (Leica, Diegem, Belgium) for normal (*i.e*. no asymmetric) cell division of the first few cell cycles (4- to 64-cell stage). Unfertilised or asymmetric eggs were discarded from the experiment and subsequently

replaced by another egg. After selecting sufficient eggs, all eggs were randomly transferred to a 48 well plate (Greiner Bio-One, Wemmel, Belgium) filled with control embryo medium. In order for a batch of eggs to be valid, a fertilisation rate of ≥ 90% and control embryo death of ≤ 10% was necessary. All experiments fulfilled both criteria.

6.2.3 Morphological analyses of the embryos

Each control and test group consisted out of 20 embryos and the experiments were replicated. Control embryos were reared in embryo solution until the end of organogenesis (96 hpf). Test groups were transferred from the embryo medium to their test solution after $5^{1/4}$ hpf, which marks the onset of organogenesis. They were kept in the test solutions until the end of the experiment (96 hpf).
At several timepoints during the period (*i.e.* $5^{1/4}$, 10, 24, 48, 72 and 96 hpf), zebrafish were morphologically analysed for several parameters including hatching, oedemas, cardiovascular development, tail malformations and facial parameters amongst others. After the last scoring (96 hpf), larvae were euthanised by means of an overdose of MS-222 (1 g/l in embryo medium).

6.2.3.1 Morphological scoring of a series of dilutions after preincubation

HLM and NADPH tetrasodium salt at concentrations of 200 µg/ml and 1.25 mM, respectively, were prepared in embryo medium and preincubated at 28.5°C for one hour. Next, the solution was either diluted 1/10, 1/20 or 1/30 . At $5^{1/4}$ hpf, embryos were exposed to control embryo medium or to one of the dilutions of the preincubation mixture throughout organogenesis.

6.2.3.2 Morphological scoring of the supernatant of the preincubation system

A solution of HLM (200 µg/ml) and NADPH tetrasodium salt (1.25 mM) in embryo medium was prepared and preincubated at 28.5°C for one hour. Afterwards, ultracentrifugation was carried out at 100 000 x g (4°C) for one hour. The supernatant was carefully separated from the pellet and used for the morphological experiments. The controls were exposed to embryo medium, whilst the test groups were exposed to either the undiluted supernatant or a dilution of the supernatant (*i.e.* 1/10, 1/20 or 1/30).

6.2.3.3 Morphological scoring of embryos exposed to trimethadione and its main human metabolite

A preincubation solution was prepared by adding 200 µg/ml of HLM, 1.25 mM NADPH tetrasodium salt and 30 mM trimethadione to embryo medium. After 1 hour of preincubation at 28.5°C, the solution was ultracentrifuged at 100 000 x g (4°C) and the supernatant was carefully separated from the pellet. The experiment consisted of 7 groups, including a negative and positive control, listed in the table

below (table 1). Each group consisted out of 20 embryos and the experiment were replicated three times.

Table 1. Overview of the groups included in the preincubation experiment with trimethadione.

Test group	Exposure
Negative control	Embryo medium
Positive control	30 mM trimethadione
Ultracentrifuged preincubation sample (UC'ed)	Supernatant of the preincubation mixture
UC'ed 1/10	Diluted supernatant of the preincubation mixture
15 mM Dimethadione (DM 15)	15 mM of the primary teratogenic metabolite
1.5 mM Dimethadione (DM 1.5)	1.5 mM of the primary teratogenic metabolite
0.15 mM Dimethadione (DM 0.15)	0.15 mM of the primary teratogenic metabolite

6.2.4 ROS measurement of the preincubation mixture

ROS in the preincubation medium was analysed to see whether ROS levels exceeded previously reported non-embryotoxic values[8]. A negative control consisted out of embryo medium, whereas a positive control sample consisted of 200 µg/ml HLM and 1.25 mM NADPH tetrasodium salt in embryo solution, after which 1 hour of incubation at 28.5°C was carried out. Two test groups were prepared similarly to the group above (UC'ed and UC'ed 1/10, Table 1), only were they subsequently ultracentrifuged at 100 000 x g (4°C). The supernatant of one of these two mixtures remained undiluted, while the other sample was diluted 1/10. The last two groups were prepared in the same manner, but trimethadione (30 mM) was added before the incubation step of 1 hour. Right before the ROS measurement, the dye was added to the solutions at a final concentration of 8.67 µM CM-H_2DCFDA. Samples were loaded in a black 96-well plate and placed in a Tecan Infinite M200 Pro (Tecan, Männedorf, Switzerland). Fluorescent values were measured after 1 hour of incubation at 28.5°C. Excitation and emission wavelengths were 493 nm and 522 nm, respectively.

6.2.5 Statistical analysis

All statistical analyses were carried out in Graphpad Prism 7 (San Diego, CA, USA). Results were considered significant at a p value ≤ 0.05. Morphological data was analysed by means of the Fisher's exact test. We chose this analytical method as cell counts in a number of the contingency tables were too low (≤ 5) and, as a consequence, chi-squared analysis was impossible. We analysed the amount of

ROS in the medium after 60 minutes by means of a Mann Witney-U test. The signal can be correlated to the amount of ROS and is expressed in the figure as relative fluorescence units (RFU).

6.3 Results

6.3.1 Validity of the experiments

The negative controls had a coagulation of less than 10% and fertilisation rate was above 90%. As such, all experiments were valid. After comparing the morphological effects of the control groups in all replicates, there was no replicate showing more or less malformation than the other replicates for any of the parameters ($p \geq 0.163$). Therefore, we pooled the data of all replicates for each experiment.

6.3.2 Embryotoxicity of a series of dilutions of the preincubation mixture

Concerning coagulation or embryonic death, there was an increase in coagulated embryos at 96 hpf in the group of embryos exposed to 1/10 diluted MAS ($p = 0.0156$). At earlier timepoints, the difference was not yet noticeable ($p \geq 0.2068$). In the 1/20 and 1/30 diluted group, there was no difference in coagulation ($p > 0.9999$).

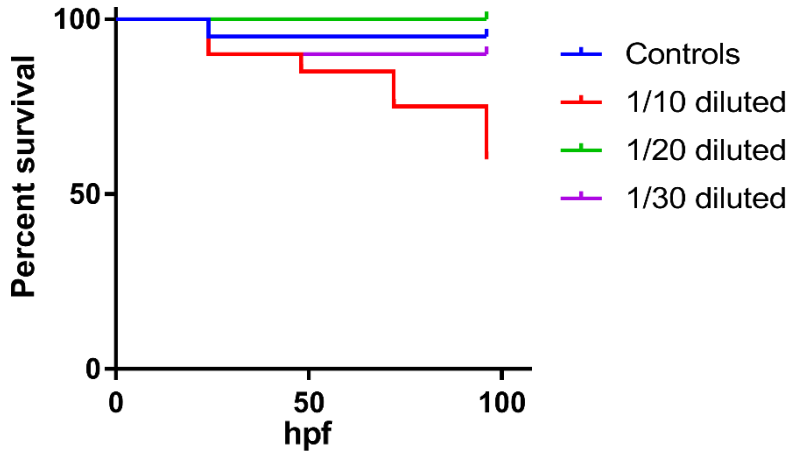

Figure 1. Survival curve of the controls and dilutions of the preincubation mixtures

Besides embryonic death, a higher amount of affected embryos was also present in the 1/10 diluted group at 96 hpf ($p < 0.0001$). This increase was first noted after 48 hpf ($p = 0.0001$), at which the amount of embryos with a yolk malformation was already increased, with a p-value of 0.0260 (table 2). The shape of the yolk was irregular and it was also enlarged compared to the controls, which might indicate a lower consumption of nutrients. At 24 hpf, no effect of the 1/10 diluted medium could be observed

yet ($p \geq 0.1528$). For the groups of embryos exposed to a 1/20 or 1/30 dilution of the MAS, no morphological events were present throughout the exposure window.

Table 2. Overview of coagulation, the amount of affected embryos and the amount of yolk malformations in the 1/10 diluted preincubation mixture compared to the controls.

	48 hpf			72 hpf			96 hpf		
	Controls	1/10 dilution	p-value	Controls	1/10 dilution	p-value	Controls	1/10 dilution	p-value
Coagulated embryos	1/60	3/60	0.6186	1/60	5/60	0.2068	1/60	8/60	0.0156
Affected embryos	2/59	16/57	0.0002	2/59	12/55	0.2066	1/59	16/52	< 0.0001
Malformation yolk	2/59	9/57	0.0260	0/59	11/55	0.0002	1/59	16/52	< 0.0001

However, as a 1/20 dilution of the metabolites might be too high and toxicity of the MAS was low in the 1/10 diluted group, an extra step, namely ultracentrifugation prior to dilution, was tested. The results of this approach are represented below.

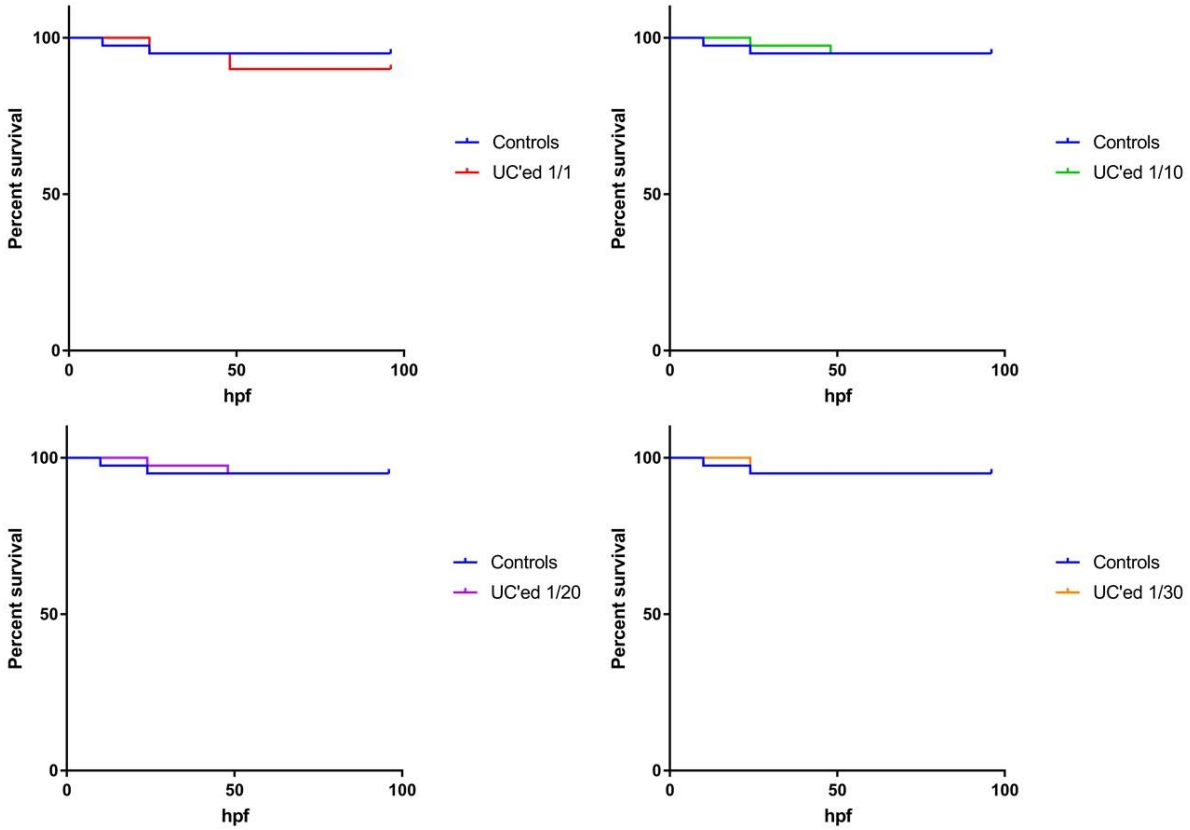

Figure 2. Survival curves for all groups. The diluted groups (1/10, 1/20 and 1/30) are shown in different graphs, as overlap of the curves made it impossible to represent the results in one graph.

6.3.3 Embryotoxicity of the supernatant of the preincubation system

At 96 hpf no coagulation was observed in any of the groups ($p \geq 0.6752$) (figure 2).

In general, the total number of affected embryos (*i.e.* the number of embryos with at least one malformation) did not differ between any test group and the controls ($p \geq 0.5941$).

Concerning the specific morphological parameters, the group that remained undiluted after ultracentrifugation showed an increase in the number of embryos with a malformation of the yolk ($p = 0.0077$). This was already the case at 24 and 48 hpf as the respective p-values were < 0.0001 and 0.0005. In this case, the yolk was irregular of shape and bigger compared to the controls, meaning a decreased consumption of the nutrients inside of the yolk. Moreover, a parameter which is not scored as such, but nonetheless obvious, was the fact that many of the embryos in this group were laying on their side instead of balancing themselves upright. Their blood circulation seemed to be slower as well, though not quantified. All other parameters in this (table 3) and all other groups did not differ from the controls ($p \geq 0.4648$).

Table 3. Overview of affected parameters in the undiluted ultracentrifugation sample group.

Affected parameter	Controls	Undiluted ultracentrifugation sample	P-value
Coagulation	2/40	4/40	0.6752
Affected embryos	1/38	6/36	0.0528
Oedema pericard	0/38	1/36	0.4865
Oedema yolk	0/38	1/36	0.4865
Blood accumulation yolk	0/38	1/36	0.4865
Malformation yolk	0/38	**6/36**	**0.0077**
Malformation heart	0/38	1/36	0.4865
No blood circulation	0/38	1/36	0.4865
Deviating shape of head	0/38	1/36	0.4865
Deviating shape of mouth	0/38	1/36	0.4865

As a result of the absence of malformations in the 1/10, 1/20 and 1/30 diluted groups, ultracentrifugation with subsequent dilution is suitable for preincubation purposes and necessary, as the undiluted group showed an increase in embryos with a yolk malformation.

6.3.4 The effect of trimethadione on zebrafish embryonic development

For the embryos that were exposed to the undiluted ultracentrifuged medium, coagulation was 100% ($p < 0.0001$). As a result, malformations could not be assessed in this group at 96 hpf. Moreover, at 72 hpf, all embryos were already coagulated and at 48 hpf, 20/60 embryos were already dead ($p = 0.0013$). At 24 hpf, however, only 8 out of 60 embryos died, which was not yet higher compared to the controls ($p = 0.5585$). P-values for all other groups at these timepoints (24-96 hpf) were ≥ 0.0573 (figure 3).

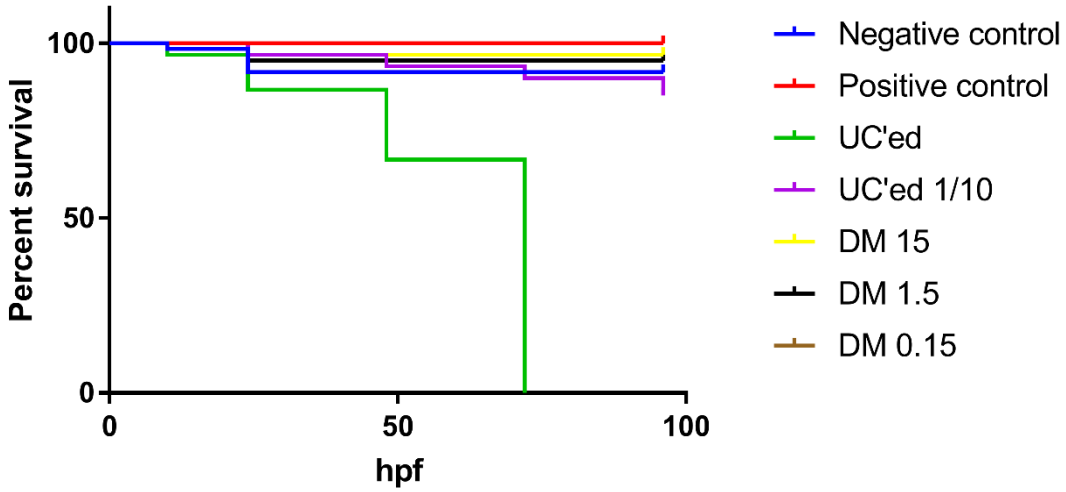

Figure 3. Survival curve for the controls and all test groups. The black bar of DM 1.5 and brown bar of DM 0.15 overlap completely.

At 96 hpf, the group exposed to the parent compound (30 mM trimethadione) without MAS showed a higher number of affected embryos than the controls ($p < 0.0001$), with almost every embryo affected (98%) and this could be observed at earlier timepoints already. At 48 hpf, approximately 30% of embryos was affected and after 72 hpf, over 50% of embryos already had a malformation. The different morphological parameters that were affected (*i.e.* significantly higher compared to the controls) at the different timepoints are represented in table 4. Eight parameters were significantly affected ($p \leq 0.0334$), with mostly problems of the cardiovascular system. After 72 hpf, of these 8 parameters, 4 were already significantly increased compared to the controls. Looking at 48 hpf, this was only one parameter. Curved tail could, however, not be analysed at this timepoint as none of the embryos in both the control and 30 MM trimethadione group hatched.

Table 4. Overview of the malformations in the group exposed to 30 mM trimethadione at 96 hpf (upper panel), 72 hpf (middle panel) and 48 hpf (lower panel).

Affected parameter at 96 hpf	Controls	30 mM trimethadione	P-value
Coagulation	5/60	0/60	< 0.0001
Affected embryos	1/55	56/60	< 0.0001
Hatching	0/55	31/60	< 0.0001
Curved tail	1/55	17/29	< 0.0001
Oedema pericard	1/55	45/60	< 0.0001
Blood accumulation yolk	0/55	7/60	0.0134
Malformation yolk	1/55	20/60	< 0.0001
No blood circulation	1/55	30/60	< 0.0001
Absence of a heartbeat	1/55	8/60	0.0334
Deviating shape of mouth	1/55	13/60	0.0011

Affected parameter at 72 hpf	Controls	30 mM trimethadione	P-value
Coagulation	5/60	0/60	0.0573
Affected embryos	8/55	36/60	< 0.0001
Hatching	21/55	48/60	0.0252
Curved tail	1/25	5/12	0.0089
Oedema pericard	1/55	34/60	< 0.0001
Blood accumulation yolk	0/55	3/60	0.2449
Malformation yolk	1/55	6/60	0.1166
No blood circulation	1/55	11/60	0.0045
Absence of a heartbeat	0/55	2/60	0.4966
Deviating shape of mouth	1/55	0/60	0.4783

Affected parameter at 48 hpf	Controls	30 mM trimethadione	P-value
Coagulation	5/60	0/60	0.0573
Affected embryos	1/55	22/60	< 0.0001
Hatching	55/55	60/60	/
Oedema pericard	0/55	18/60	< 0.0001
No blood circulation	0/55	4/60	0.1199

At 24 hpf, no difference in affected embryos could be observed (p = 0.8648).

For the highest metabolite group (15 mM dimethadione), the difference in affected embryos at 96 hpf was more subtle (8/50 embryos, p = 0.0323) and no difference could be observed at 72 hpf or earlier timepoints (p ≥ 0.7532). The other groups did not differ from the controls.

6.3.5 ROS measurement in the preincubation system

All groups were compared to the controls and differed significantly from them (p ≤ 0.0079) (figure 4). For the group of HLM that were ultracentrifuged with subsequent 1/10 dilution of the supernatant, the amount of ROS in the medium was even lower compared to the controls (p = 0.0079). For all other groups, there was an increase in ROS. The amount of ROS was highest in the positive control (not ultracentrifuged HLM and NADPH) The lowest signal was found in the group of which the supernatant was diluted 1/10 after ultracentrifugation. ROS levels in this group were below the value of the control which consisted of only medium. The values of the other groups were situated between the control group and the positive controls.

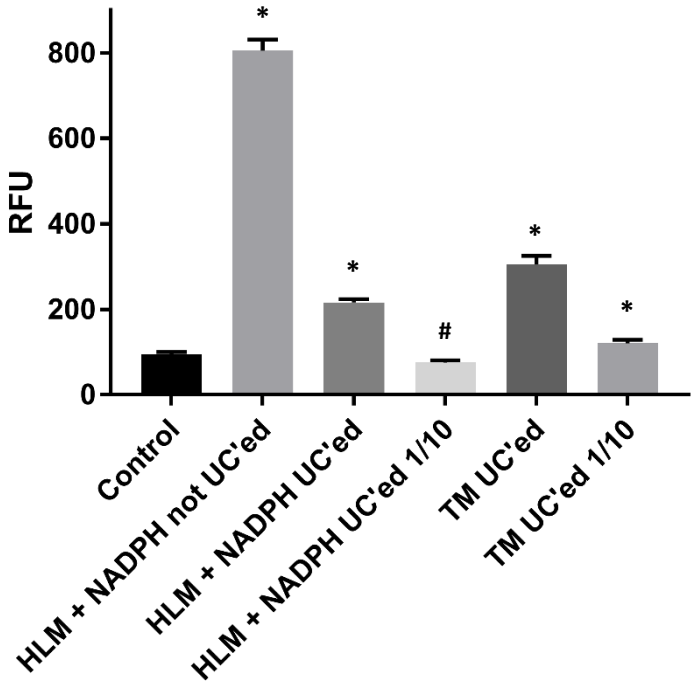

Figure 4. Relative fluorescence values of all groups tested. * Indicates an increase of ROS in the medium compared to the controls. # Indicates a decrease OF ROS in the medium compared to the controls.

6.4 Discussion

This study showed that preincubation of HLM and NADPH (administered as a single dose) with subsequent ultracentrifugation and dilution of the supernatant is non-embryotoxic. To achieve the highest amount of metabolites in the mixture, it is advised to use the 1/10 dilution approach, rather than 1/20 or 1/30. On one hand, dilution of the samples is necessary to reduce the toxicity of the MAS, as no dilution leads to coagulation[8]. On the other hand, ultracentrifugation is required to completely resolve toxicity. When we did not combine both steps, an increase in coagulation and in the amount of affected embryos was present at the end of organogenesis, which expressed itself as an increase in yolk malformations. The embryos in the affected groups developed slower and were lagging behind compared to the other groups. Larvae were unable to properly balance themselves and heart rate and blood circulation in these embryos seemed slower. This has previously been reported and is linked to developmental stage[9].

As no effect was seen in the group exposed to a 1/10 diluted supernatant, this option seems to be ideal for further testing. Also, the advantage of 1/10 dilution over a 1/20 dilution is that the parent compound and its metabolites are less diluted. Thus, exposure will be higher and the results are more relevant for extrapolation to *in vivo* data and human developmental toxicity.

Therefore, we tested this approach with a tool compound. Trimethadione was selected, as it has been proposed as a possible proteratogenic agent in mammals[10,11]. Moreover, a range finding study in zebrafish embryos was carried out earlier by Weigt *et al.* (2011)[10]. Based on this latter study, we expected 50% of affected embryos in the parent group, as 30 mM was close to the EC_{50} value calculated by Weigt *et al.*[10]. However, in our study 56/60 embryos were affected. Weigt *et al.* exposed embryos from 2.5 until 72 hpf, whereas our exposure started slightly later ($5^{1/4}$ hpf, namely at the onset of organogenesis) and lasted throughout organogenesis (*i.e.* 96 hpf). As a result, we exposed the zebrafish larvae almost 24h longer than Weigt *et al.* In fact, when we looked at the amount of affected embryos at 72 hpf, 36/60 embryos were affected, approaching the EC_{50} value determined by Weigt *et al.* Therefore, exposure length is the factor causing the difference and hence, prove has once more been given that exposure throughout organogenesis is pivotal.

We also noted that addition of 30 mM trimethadione in the pre-incubation system caused 100% lethality whereas the same mixture without trimethadione caused no significant increase in coagulation. Moreover, its human metabolite dimethadione did not show the malformations which one would expect based on the study of Weigt *et al.*[10]. In this latter study, the authors stated that zebrafish are capable of biotransforming the proteratogenic parent compound, trimethadione, to the teratogenic mammalian metabolite (dimethadione). However, we only noted a small increase in affected embryos at the highest dimethadione concentration and no effects at the lower dimethadione

concentrations. This suggests that the embryos are unable to biotransform the parent compound[12] into the toxic human metabolite. It is more likely that zebrafish embryos are either affected by the parent compound or that they are biotransforming the parent compound into a different metabolite that is embryotoxic for zebrafish embryos. However, this needs to be confirmed by bioanalysis (e.g. LC-MS analysis) of the medium and preferably of the internal concentrations of both tri- and dimethadione. 30 mM trimethadione alone (without pre-incubation system) did not cause any coagulation and as a result, there seems to be a synergistic effect of the toxicity caused by the compound and the toxicity of the undiluted supernatant. Concerning the diluted supernatant, we noted no significant effects on zebrafish embryonic development and embryonic death was absent as well.

This is interesting and strengthens the fact that undiluted supernatant of MAS is not suitable as a preincubation system and might skew the results when a (pro)teratogenic compound is added to it. As a result of these findings, we were interested whether ROS in the medium could play a role in this synergistic effect. We previously described increased ROS levels during coincubation with HLM and NADPH[8], be it at non-toxic concentrations. Nonetheless, we wanted to rule out ROS as trigger for the synergistic effects. Our data showed that ROS is not responsible for the toxicity, as ROS levels in all mixtures tested were lower compared to the positive control, which was a mixture of HLM and NADPH without ultracentrifugation, which in our previous study proved to be non-toxic[8]. Thus the synergistic effect cannot be explained at this moment.

In conclusion, despite the fact that in hindsight trimethadione appears to exert teratogenic effects rather than proteratogenic effects in zebrafish, ultracentrifugation and subsequent dilution of MAS prior to exposure of zebrafish embryos is not embryotoxic. This needs to be further validated with other compounds and bioanalysis in the future.

6.5 Acknowledgements

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Chapter 7: General discussion and future perspectives

After several years of research, multiple factors contributing to the toxicity of the mDarT have been identified, including rearing temperature and protease inhibitors amongst others. Therefore, the original testing strategy of Busquet *et al.* from 2008[1] has been modified in this thesis. This ultimately led to an altered approach in which preincubation was preferred over coincubation in the metabolic zebrafish developmental toxicity assay or mZEDTA. As a guidance throughout this discussion, figure 1 represents the flowchart of the doctoral thesis and table 1 indicates the major differences between the mDarT and the mZEDTA.

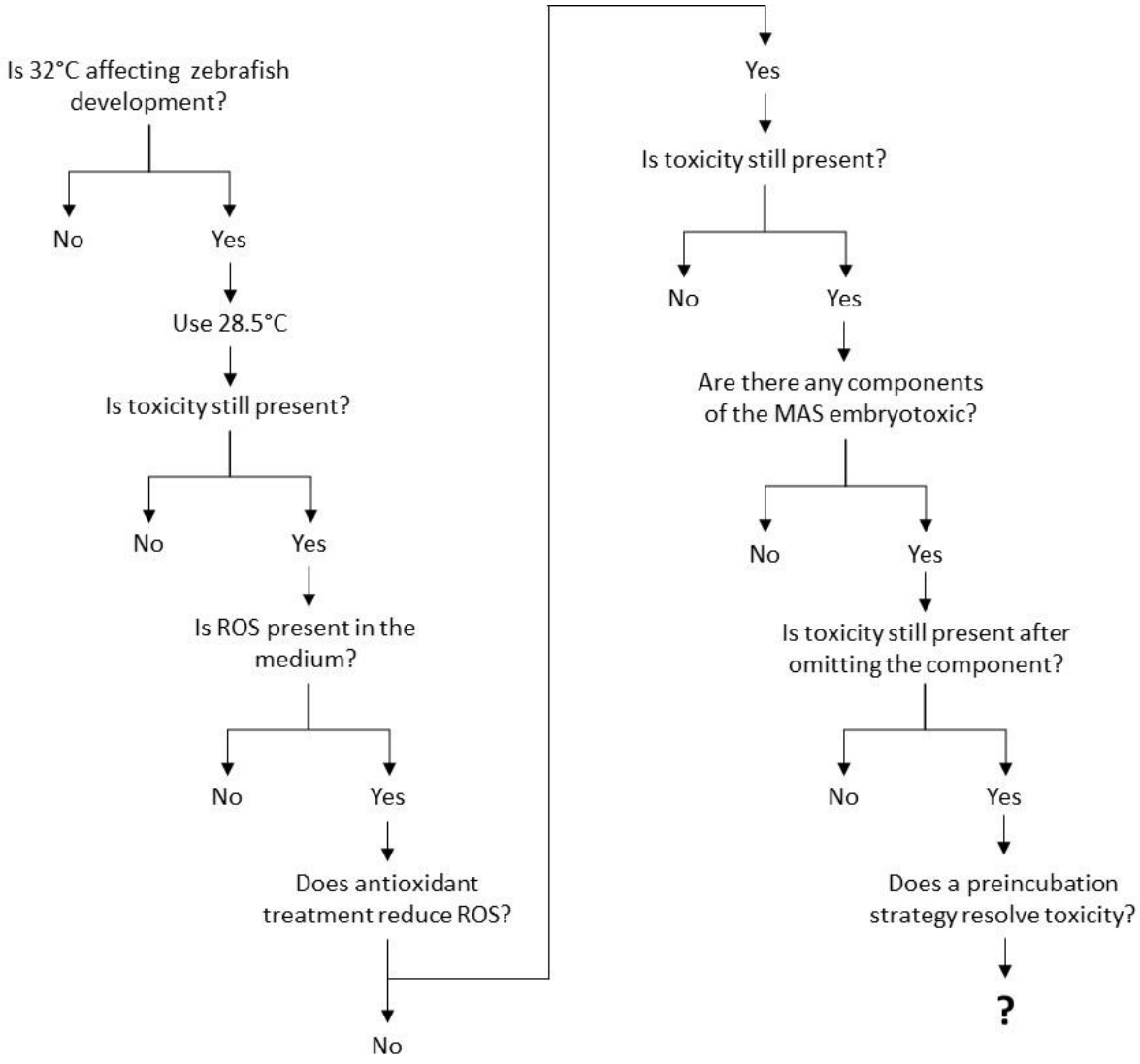


Figure 1. Final flowchart of the doctoral project.

Table 1. Overview of the major differences between the mDarT and the mZEDTA

Factor	mDarT	mZEDTA	Reasoning
Compound exposure	2 - 3 hpf	5.25 - 96 hpf	Exposure during organogenesis
Rearing	2 - 48 hpf	2 - 96 hpf	Final analysis should be carried out at the end of organogenesis
Temperature	32°C	28.5°C	Optimal for zebrafish development and CYP activity guaranteed
Analysis	Morphology	Morphology + bioanalysis	Concentration in rearing medium ≠ internal concentration

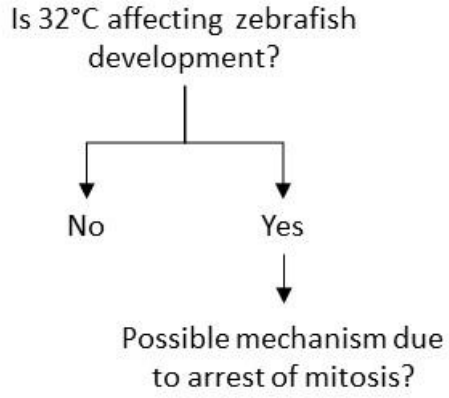
In theory, these modifications (table 1) should reduce the rate of false positive and false negative results in the mZEDTA compared to in vivo data, as exposure is optimised. As an example, in chapter 5 we described the effects of short vs. longer exposure (respectively 1 and 70 hours) to dimethadione, which clearly shows that prolonged exposures can aggravate the outcome. As continuous exposure is also carried out in vivo, our system mimics the in vivo situation better than the mDarT. Not only is continuous exposure during the entire period of organogenesis possible in the mZEDTA, renewal of the medium can be carried out for products that are less stable in solution. In the mDarT, however, only one hour of exposure is carried out followed by rearing until 48 hpf in a control embryo medium. We also showed that continuous rearing of embryos at 32.5°C is too high for normal embryonic development. However, in a preincubation setting, the preincubate can be prepared at whatever temperature is desired and the supernatant can subsequently be coincubated with the embryos at 28.5°C. A last factor of importance is the inclusion of bioanalytical data in our setting. Not every compound has the same rate of uptake in the zebrafish embryo assay and thus, determination of medium, but also internal, concentrations can shed a light on the uptake of the parent compound and its metabolites. In the following subchapters, we will discuss the above findings further.

7.1 Metabolic capacities of zebrafish embryos

One might ask whether there is a need for an exogenous metabolic activation system in the mDarT. It is the case that several research groups suggest that zebrafish embryos are capable of biotransforming compounds to their metabolites[2]. Before mid-blastula transition, all factors that are involved in developmental processes in the zebrafish embryo are from maternal origin. It is during mid-blastula transition that the zygotic genome is activated and this is also the case for CYPs. Maternal transfer of CYP mRNA occurs but after 2 hours post fertilisation, the mRNA has been degraded. This leaves a gap

between 2 hours and 5 hours post fertilisation at which CYP expression levels are very low[3]. It is only after 72 hpf that most of the CYPs involved in the metabolism of drugs have increasing expression levels[3] This was also confirmed by a study within our research group (paper in preparation). Other work within our group also indicates a limited to absent CYP-mediated metabolic capacity of zebrafish embryos[4–7]. Therefore, indications that metabolism is either absent or too low to have relevance for developmental toxicity screening are present. For as long as no conclusive data is available on the presence of sufficient CYP-mediated metabolism throughout the entire period of organogenesis, inclusion of a MAS is pivotal for correct interpretation of results from the zebrafish embryo assay.

7.2 The role of temperature on zebrafish embryonic development



In our study, we observed a positive correlation between increasing temperatures and teratogenicity. For the controls (28.5°C) and the lowest test group (30.5°C), no effects were present in the developing embryos. From 32.5°C onwards, malformations started to occur (mainly malformations of the tail) and they increased with increasing temperatures. From 34.5°C onwards, coagulation was present and was very high (approximately 80%) in the group exposed to the highest temperature (36.5°C). For the latter group, several parameters of abnormal development were increased. These results are in line with previous reports in other species[8]. In mammals, teratogenic effects of increased embryonic temperatures (due to fever or decreased embryonic blood flow[9]), can be mild to severe (e.g. anencephaly)[10,11]. Moreover, the reported increase in coagulation of zebrafish embryos at the highest two rearing temperatures is in agreement with an increase in stillbirths in mammals[10,11]. Even though zebrafish are poikilothermic animals and adults can stand a wide temperature range (6.2 – 41.7°C), zebrafish embryos, thus, seem to be more sensitive to deviations of temperatures from the optimal[12]. In mammals for example an increase of embryonic temperature of 1.5 – 2.5°C is known to cause increased rates of resorption, abortion and malformations, with the nervous system being the most sensitive tissue type[13,14]. Effects are probably due to immediate arrest of mitosis and cell death of cells

in mitosis leading to e.g. microencephaly. Moreover, micro disruptions of the cardiovascular system could lead to haemorrhages and oedema[13,14]. All of these effects were also present in our study with zebrafish embryos, especially in the highest temperature groups, indicating a possible common mechanism. As cell death as a result of arrest of mitosis plays a role in the malformations that occur, it is not surprising that zebrafish early life stages are more sensitive than their adult counterparts. During embryonic development, mitosis is constantly occurring leading to growth of the embryo/larva. For most tissue types in the adult animal, the rate of proliferation of cells is decreased and thus, limited periods of exposure to more extreme temperatures can be tolerated.

As an increase in embryos with a malformation, as well as an increase in tail malformations was present in the group exposed to 32.5°C, it is questionable why research groups that are exploring the use of a MAS have been coincubating MAS with zebrafish embryos at a temperature of 32°C[1,15,16]. The first group to coincubate zebrafish with a MAS was Busquet *et al*. (2008). Their rationale behind the selection of 32°C as a coincubation temperature was based on a review of Kimmel *et al.* (1995). In this review, it is stated that 32°C is tolerated by zebrafish[17]. On the other hand, Busquet *et al*. hypothesised that enzymatic activity of the liver microsomes will be guaranteed even though 32°C is not the normal physiological temperature for CYP enzymes in mammals. Interestingly, there have been older reports claiming that the temperature dependence of CYPs is rather limited, with high metabolic activity for a rather large range of temperatures[18,19]. Also, more recent research in our group showed that CYP activity is not decreased at 28.5°C compared to 37.5°C[4]. CYPs are enzymes that can be found in a wide range of species and even in some unicellular organisms such as algae[20] and in bacteria[21], including mitochondria of higher organisms[22]. This indicate a distinct biological relevance for normal (cell) functioning but also the capability of enzyme activity under a range of circumstances such as different environmental temperatures. CYP119 for example, a CYP constitutive to *S. Solfataricus* is a highly thermostable CYP with a melting temperature that is approx. 40°C higher than most other CYPs[23]. From research within our research group, mammalian CYPs seem to have a certain degree of temperature tolerance as well. This is likely due to several determinants such as an increased hydrogen bonding, ionic stabilization of α-helixes, tighter packing of the protein core, increased number of salt links and salt-link networks, increased number of disulphide bonds, decreased conformational entropy of the unfolded state, and increased aromatic stacking interactions[23]. Thus, CYPs are probably owing their slightly increased temperature tolerance to a combination of some of these factors.

Concerning development at rearing temperatures of 32°C, reports on morphological effects of rearing temperature for zebrafish embryos are scarce. In a study by Schirone and Gross (1968), no morphological effects were reported for temperatures ranging from 28-34°C. However, in this study, exposure was short (until the closure of the neuropore, which happens around 9 hpf)[24] and as a result,

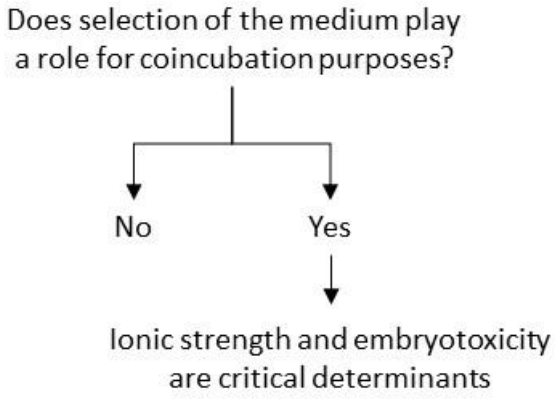
morphological scoring was not carried out in this study. Another study in which temperature and effects on embryonic development were reported was a study from Schnurr *et al*. (2014)[25]. Here, zebrafish embryos were reared at several (increased) temperatures up until hatching. Morphological scoring consisted solely out of hatching success and lethality, without scoring of any other morphological parameters. Thus not only was the number of parameters limited in both studies, exposure was also shorter. Also, it is well known that heat as a teratogen is dependent on dose (*i.e.* temperature), duration (*i.e.* duration of the increased embryonic temperature) and timing (*i.e.* sensitivity to heat is not identical throughout development). Therefore, a detailed report on the effects of temperature during the entire period of embryonic development has not yet been described but is crucial information for the rearing temperature selection in the mDarT.

It is also known that different embryonic temperatures have an effect on developmental speed. This is due to a decrease of the cell duplication time. The cell duplication time can be reduced at higher temperatures as the rate of DNA replication is reduced per replication fork. The distance between different initiating points, however, does not alter depending on temperature[26]. As the rate of cell division is a direct measure for the rate of organ and cell growth, the effect of increasing temperatures has an accelerative effect on development. As already discussed above, there are limitations to this acceleration as arrest of mitosis occurs at higher temperatures that are more distant from the optimal developmental temperature.

In the two previously mentioned studies, developmental speed was increased with increasing temperature, with the fastest development around 32-34°C[24,25]. This is in line with our findings as we noted an increase in hatching rate for the higher temperatures. However, there was a steep cut-off for the embryos reared at 36°C, as they drastically failed to hatch and ultimately died. Even though staging was not within the scope of this study, absence of a parameter for definite staging is a downside of this study. Hatching is mere an indicator for developmental speed, but parameters such as number of somites, length measurement or heart rate are more reliable staging indices and could have been easily included.

Nonetheless, we describe into detail the effects of increased rearing temperature on zebrafish embryonic development and its consequences for coincubation of zebrafish embryos with a MAS. We also advice to decrease the temperature for coincubation of a MAS with zebrafish embryos to 28.5°C, as CYP activity is guaranteed, as well as normal embryonic development.

7.3 Suitable media for the mDarT



The temperature experiments described above were carried out in a medium which is reconstituted fresh water that is similar to the conditions in which adult zebrafish are kept. It is a non-toxic medium for both zebrafish and their embryos[27]. However, our results show that CYPs are no longer active when they are incubated in the embryo medium. CYP activity assays with liver microsomes are performed most often with a potassium phosphate buffered medium, which proved to be toxic for zebrafish embryos. Thus a range of several other media was tested for both CYP activity and normal embryonic development.

The fluorogenic ethoxyresorufin-O-deethylase (ERDO) assay was used for testing the media and has a very high specificity for CYP1A activity[28]. As such, the assay is not representative for the total CYP activity but rather/mainly CYP1A activity. However, as all major drug metabolising CYPs are located in the ER, and have the same mechanism for activity, the impact is expected to be the same. Moreover, we only selected the media showing the highest EROD activity (*i.e.* the media with a comparable CYP1A activity to the standard potassium phosphate buffered medium) for further morphological evaluation. Concerning the activity of the media, only a select number showed identical CYP activity to the control. Two out of those four media were variations to the standard buffer. One of them was supplemented with Instant Ocean sea salts and the other was supplemented with embryo solution. Both supplementations were based on existing incubation media. However, when they were tested for their activity without the addition of potassium phosphate buffer (instant ocean sea salts), or very low supplementation of potassium phosphate buffer (0.001M PPBM in embryo solution), only a very low signal could be measured. Therefore, supplementation of these media seems to be required for activity of the CYP enzymes. The other two media showing no difference in activity compared to the control are both consisting of 0.1M Tris-HCl buffered medium in either embryo solution or reverse osmosis water. The signal of 0.1M Tris-HCl in Instant Ocean sea salts was lower compared to the two others and the controls. A variety of other media and modifications/supplementations to the hereabove described media all proved to be less suitable in terms of CYP activity. However, as we tested several

concentrations for both Tris-HCl as well as potassium phosphate buffer, we did not include multiple concentrations for the other buffers and merely tested a concentration found in literature, which was a downside to this study. However, the concentration will probably not have been of that big an influence that one of the other media could achieve an activity comparable to the activity of the 0.1M potassium phosphate buffer.

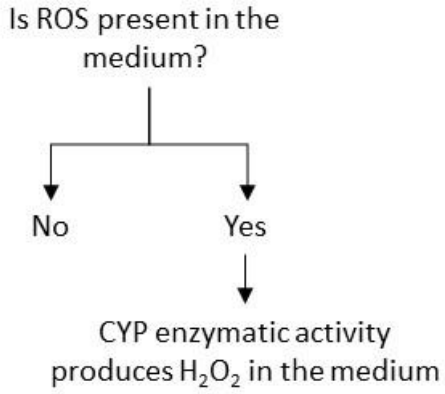
Morphological examination of the control medium, as well as the four media with sufficiently high CYP activity revealed that only 1 medium was suitable for coincubation purposes with zebrafish embryos, namely 0.1M Tris-HCl buffer in embryo solution. Embryonic death did not occur in the controls, which were reared in Instant Ocean sea salts, however, this medium showed no CYP activity and was, thus, not suitable for coincubation purposes. The other media that were morphologically tested were all embryotoxic, as coagulation occurred before the end of organogenesis in all embryos. For 0.1M Tris-HCl in reverse osmosis water, there might be a lack of necessary ions for zebrafish embryonic development. Whilst for the two potassium phosphate buffered media, the concentration of either potassium or phosphate will interfering with normal development. To our knowledge, there are no reports on minimal needs and toxic levels concerning (specific) ions, but this seems to be the cause for the mortality.

As a consequence, 0.1M Tris-HCl buffered medium was considered to be the optimal medium for coincubation purposes. This is not surprising, as other research groups are also using this buffer[1,15,16]. A difference between these coincubation studies and our fluorogenic assay, however, is the protein concentration of the human liver microsomes. Whereas in our study we used 200 µg proteins/ml, two studies used 700 µg/ml[1,16] and the other study used a protein concentration of 0.707 nmol/ml[6]. No explanation on the selection of the concentration of proteins was provided by the authors of the three studies. On the other hand, after optimisation in our lab, we found the optimal protein concentration for microsomal activity to be 200 µg/ml[29], which is why we applied this concentration in our studies.

After defining the optimal coincubation medium, coincubation of HLM and NADPH with zebrafish embryos reared in this medium was performed. Toxicity was still present, as zebrafish embryos died before the end of organogenesis. Therefore, we also looked at short exposure (*i.e*. 1 hour) compared to a longer exposure period (*i.e.* 70 hours). As a tool compound for this, dimethadione was chosen. Results from this study clearly showed that longer exposure caused more effects. This is not surprising, as developmental effects are caused by a certain dose, as well as a certain duration. Moreover, not every organ system develops at the same moment, thus the timing of exposure might also play a critical role. The results of our study with dimethadione are in line with the principles of teratogenesis and agree to the available literature. Examples of this are the effects (or rather the absence of effects) found in a study by Pruvot *et al*. (2012)[30]. In this study, no teratogenic effects could be addressed to

caffeine, carbamazepine, lithium chloride or pentobarbital. However, reports from several other studies correctly identified these compounds as teratogens whilst the concentrations tested were within the same magnitude[31–34]. To highlight one of these compounds, caffeine caused no apparent effects in the study by Pruvot *et al*. (2012), but the exposure window only started at 48 hpf. In a study by Selderslaghs *et al.* (2012) exposure already started within 2 hpf and effects were apparent (e.g. delayed hatching and malformations of the cardiovascular system)[32]. Teixido *et al.* exposed zebrafish embryos from 4 hpf onwards and even though thorough morphological examination was not carried out in this study, they mention that certain analyses could not be carried out due to gross morphological abnormalities[34]. These results from literature, together with our result for dimethadione exposure, stress the need for a continuous exposure to the drug and its metabolite during the entire period of teratogenesis, as is the case in mammalian developmental toxicity testing.

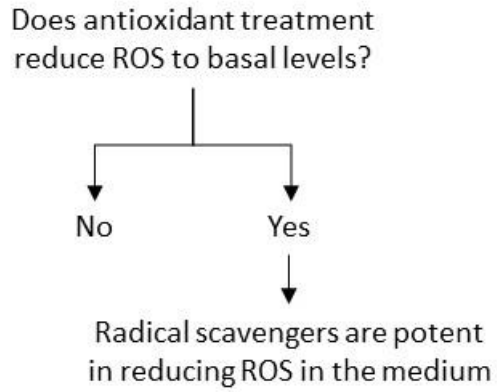
7.4 ROS in the rearing medium



Because toxicity was still present and reactive oxygen species (ROS) are produced during the enzymatic reaction of CYPs, the effects of ROS in the medium on zebrafish embryonic development were investigated. Concerning the ROS probe, we used a general ROS probe (CM-H_2DCFDA). This probe, which is a chloromethyl derivative of H_2DCFDA, can be trapped in the cell where it subsequently emits a fluorescent signal when it comes into contact with ROS[35]. The supplier suggests on its website that trapping in the cell by means of cleavage of the probe by intracellular esterases is required before a fluorescent signal can be obtained[35]. This is in fact not the case as fluorescence could be produced and measured in our study in the absence of cells. Therefore, the interaction of ROS with the probe can be used as an indicator for ROS levels in a cell-free medium such as in our study.

By means of this probe, we were able to detect ROS formation as a result of microsomal activity. What was interesting is that ROS was also detectable in the absence of the necessary co-factor (NADPH) for cytochromal activity. Thus, not only CYP activity produces ROS during coincubation, some other

components of the microsomal mixture also produce radicals to a certain degree. Moreover, mixtures in which the NADPH regenerating system was included had lower ROS levels compared to other mixtures, indicating that NADPH can play a protective role in terms of oxidative stress. In this case, NADPH reacts with glutathione-S-transferase[36], present in the microsomes, to reduce the amount of free radicals.

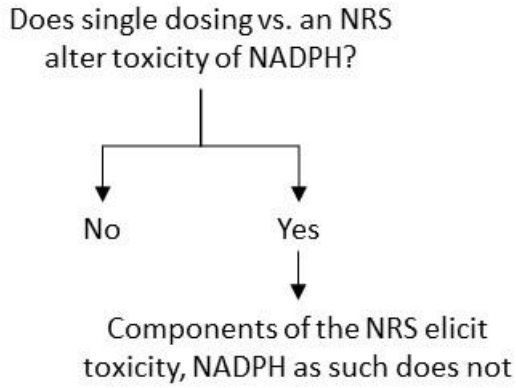


After adding antioxidants (rosmarinic acid and gallic acid) to the coincubation medium, ROS could be significantly reduced. Selenium-methionine, on the other hand, was incapable of reducing the oxidative stress, even at its highest dose. This is not surprising, as Selenium-methionine is part of the glutathione-peroxidase enzyme system[37] and in a cell free medium, antioxidants altering pathways are incapable of exerting an effect. Gallic acid (GA) is a radical scavenger[38] and its antioxidant capacity is thus not dependent on other processes, such as is the case for selenium-methionine. Rosmarinic acid is an agent that is also a radical scavenger[39]. Moreover, it prevents cell damage by preventing lipid peroxidation of membranes. However, its antioxidant capacities in our study are most likely due to its scavenging of radicals in the medium. For the next step, GA was chosen to be coincubated with HLM and NADPH. But first, safety of GA to developing zebrafish embryos had to be investigated, as GA was reported to cause detrimental effects of developing chicken embryos[40,41]. The effects seen in this study were a result of autoxidation of GA, with a prooxidative effect as a result[42]. When coincubating GA with zebrafish embryos, no effect of GA on the development of zebrafish embryos could be found, in contrast to the studies in chickens. Therefore, we also coincubated HLM, NADPH and GA with zebrafish embryos. However, no positive effects of GA supplementation could be captured. Therefore, ROS do not seem to be the major trigger of toxicity.

A limitation in our study design is the fact that we only measured ROS in the medium, whilst no internal ROS concentrations were determined in the embryo. In a recent study, ROS has been determined inside embryos after exposure to CUO nanoparticles[43]. However, GA was preincubation with HLM and NADPH 1h prior to exposure to the zebrafish embryo resulted in negligible ROS levels in the medium.

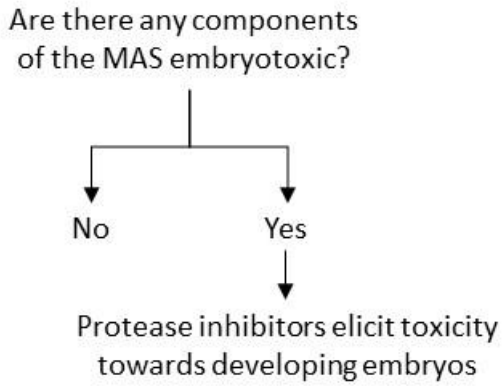
Thus, the concentration of ROS that could have entered the embryo, assuming ROS can freely diffuse through the chorion into the embryo, would be non-relevant.

7.5 Different administrations of NADPH give different toxic effects



Whereas several research groups[1,15,16] used a single dose of 1 mM NADPH as co-factor for CYP activity, we previously used an NADPH regenerating system (NRS). In this thesis we showed that the NRS is embryotoxic causing lethality before the end of organogenesis. The components of NRS were glucose-6-phosphate, glucose-6-phosphatedehydrogenase (G6PD), $MgCl_2$, $NADP^+$ and sodium citrate. There are no reports of sodium citrate and $MgCl_2$ being toxic at the concentrations of the NRS for zebrafish embryos[44]. Thus the toxicant is probably the glucose-6-phosphate or its dehydrogenase. Reports on deficiencies and alterations of this pathway are described both in zebrafish and other species as well[45–47] and thus it is plausible that it might play a role as well in the embryotoxicity that we observed. However, in our case it is not due to a deficiency in G6DP, but probably to an excess of this enzyme or its substrate. Therefore, we tested a different method to include NADPH, namely as a single-dose of its tetrasodium salt. However, exposure in those experiments was short as toxicity of the MAS was an issue[1,15,16]. This could lead to misclassification of the teratogenic potential as crucial exposure windows might be missed. In our study, no difference in CYP activity could be found between the NRS and a single dose of NADPH. Moreover, NADPH did not seem to cause any embryotoxic effects on the developing zebrafish embryos, whereas the embryos exposed to NRS showed severe malformations. This also means that in our study, as well as in the abovementioned studies, toxicity is not due to this co-factor NADPH.

7.6 Toxicity of the MAS



We morphologically examined the components that are included in a microsomal preparation (*i.e.* PI, sucrose, phosphate buffer and EDTA). As expected from a literature search, the PI was the main component causing toxicity, as some of the protease inhibitors in this kit have been linked to embryotoxicity earlier[48,49]. Other components were negative or only showed a synergistic effect in combination with PI.

Subsequently, we prepared rat liver microsomes with, and also without, PI and tested whether there would be any difference in both activity and toxicity. Omitting the PI did not alter CYP activity in the EROD assay. Also, embryotoxicity was not reduced when coincubating the PI-free rat liver microsomes with zebrafish embryos, which is striking. This means that the microsomes themselves are causing toxicity of the MAS. This is of pivotal importance, because as a result coincubation of HLM and zebrafish embryos throughout the span of organogenesis is impossible in its current form.

Finally, the liver microsomes that we prepared for this study were derived from a fresh rat liver and when we investigated CYP activity by means of an EROD assay, the activity was higher than when HLM were used. The post mortem decrease in CYP activity may explain the lower CYP activity in man, as the rat liver was immediately removed after euthanasia and kept on ice. It is well-known that CYP activity drastically decreases post mortem[50].

7.7 MAS in other *in vitro* toxicology test systems

Whereas embryotoxicity of the liver microsomes is an issue in the zebrafish embryo assay, strangely enough it is not in the FETAX. Several studies have been carried out in which the FETAX was combined with MAS[51–53]. In these studies, coincubation with liver microsomes was carried out throughout organogenesis (*i.e.* 96 hours of exposure) without toxicity of the MAS towards the developing frog embryos. Remarkably, also liver microsomes were used as MAS. Moreover, an NADPH regenerating system (NRS) was included, rather than a single dose of NADPH. We showed that an NRS is toxic for

zebrafish embryos whereas frog embryos do not suffer from exposure to NRS. Thus, there seems to be a significant difference in sensitivity to MAS and its cofactors between zebrafish and Xenopus embryos. Up until now, the reason for this difference remains unclear. Whether this is a zebrafish-specific effect or common to Cyprinidae is also unclear, as no reports exist to our knowledge on coincubation of a MAS and other fish from this family (e.g. carps, fathead minnows). Another striking fact is that the medium in which Xenopus embryos are coincubated resembles the embryo solution for rearing zebrafish, yet activity of the microsomes is reported in the FETAX[51,54,55], although no concentrations of the parent compound and the metabolite were determined, whereas it is not in the mDarT (see data Chapter 4). One of the major differences is the concentration of monovalent ions (NA^+ and K^+; see table 2). As ionic strength might play an important role in enzyme activity, it is possible that FETAX solution allows a certain degree of CYP activity, whereas embryo solution in the zebrafish embryo assay lacks ionic strength for CYP activity. This said, FETAX solution is not suited for rearing zebrafish, as high levels of monovalent ions cause lethality (data not shown) and the issue of zebrafish embryotoxicity of the MAS is also not resolved.

Table 2. Comparison of embryo solution and FETAX solution

Molecule	Embry solution	Fetax solution
$CaCl_2$	0.294	0.015
$CaSO_4$	/	0.060
$MgSO_4$	0.123	0.075
NaCl	/	0.620
KCl	0.005	/
$NaHCO_3$	0.059	0.096

Values are expressed as g/l

Another system in which MAS is applied is the Ames test for mutagenicity. In this test, bacterial colonies appear, indicating growth, even though they are exposed to S9 fraction for approximately 48 hours[56]. No toxicity of MAS to the bacteria has been reported, thus indicating that toxic effects of MAS were also not an issue in the Ames test whilst it is lethal for zebrafish embryos (data not shown). Therefore, zebrafish are probably highly sensitive to any MAS system for a reason yet to be discovered and a preincubation strategy with dilution of the MAS (e.g. by means of ultracentrifugation and subsequent dilution of the supernatant) seems the only possible solution for coincubation of zebrafish for developmental screening.

7.8 Rat versus human liver microsomes as MAS

Strangely, rat liver microsomes (RLM) are the MAS of choice for the mDarT in several groups[1,2,16] and also for the FETAX[53]. However, there is proof that the metabolites in the rat are not necessarily the same as in humans[57–60]. Diazepam is mainly metabolised into temazepam and nordiazepam but also oxazepam which in humans, monkeys and dogs. In rats, oxazepam is not formed and that the principal metabolite is 4'-hydroxy diazepam (which was quickly glucuronidated)[57]. But also for phase 2 metabolism, there is proof that species differences in metabolization might influence clearance of a drug with a subsequent effect on developmental toxicity. An example of such a drug is sulphadimethoxine, which is in most species, including humans excreted to an extent in the urine after glucuronidation (N^1- or N^4-glucuronide) of the compound. In rats, excretion is lower and glucuronidation is not present. In fact, the metabolite formed by rats is not an N-glucuronide but rather a sulphate derivative leading to a decreased excretion in the urine which means longer exposure to higher concentrations of the compound.

As the zebrafish embryo assay is used for human risk assessment, we opted to use human liver microsomes in order to gain a metabolic profile that is relevant for man. However, also this MAS has limitations, as other enzymes than CYPs can be involved in the bioactivation of compounds. This was illustrated by Klüver *et al*. (2014), who studied the effect of allyl alcohol in adult and embryonic zebrafish[61]. Allyl alcohol has a highly reactive and toxic metabolite (acrolein). There was a significant difference in ally alcohol toxicity when comparing adult zebrafish to embryos, with the latter having a higher LC_{50} value. This is due to the fact that the responsible enzymes for metabolization of allyl alcohol, alcohol dehydrogenases, are immature in zebrafish embryos and thus no toxic metabolite could be formed. There is no role for CYPs in the metabolism of allyl alcohol showing that a negative result in the mDarT does not mean that the compound is safe. Another example of teratogenicity in which CYPs are not involved is the toxicity of ribavirin. This compound causes negative effects on developing mammalian embryos due to an accumulation of ribavirin in the erythrocytes. However, the compound is categorised as false-negative in the zebrafish assay. This can be explained by the fact that zebrafish possess nucleated erythrocytes, which are capable of hydrolysing the triphosphate derivative into ribavirin again[33].

The above clearly shows that the zebrafish embryo assay needs further characterization before it can be considered to replace one of the *in vivo* mammalian developmental toxicity studies.

7.9 Evaluation of a pre-incubation strategy for the mDarT

Our study showed that preincubation of MAS at a concentration of 200 µg/ml with subsequent incubation of zebrafish embryos in the supernatant (after ultracentrifugation) was still embryotoxic. Also direct dilution of the preincubate up to 1/10 did not resolve the problem. A 1/20 dilution was non-toxic, but a quick screening using the EROD-assay showed that the generated metabolite levels are too low to be relevant for teratogenicity screening (data not shown). However, when combining the two approaches (*i.e.* ultracentrifugation of the preincubation mixture followed by a 1/10 dilution of the supernatant) no embryotoxicity was observed. As a proof-of-concept for the bioactivation potential of these optimized MAS conditions, we used the mammalian proteratogen trimethadione. However, incubation of this compound with zebrafish embryos at 30mM appeared to be more embryotoxic than when exposing the embryos to the 1/10 diluted supernatant of the MAS. Furthermore, exposure of the embryos to it human teratogenic metabolite dimethadione did not result in embryotoxicity or only very mild effects at very high concentrations. This suggests that trimethadione is a teratogenic compound in zebrafish and does not require bioactivation to exert its teratogenic potential. This is in line with a study in chicken embryos with this compound [62], but is in contrast to data of Weigt *et al*. (2011)[2], who suggested that the teratogenic effects of trimethadione in zebrafish embryos are caused by bioactivation of the compound by CYPs. As such, the authors claimed that zebrafish are capable of metabolising the compounds to such an extent that sufficiently high metabolite levels are achieved, causing the observed malformations. However, exposure in this latter study was carried out until 72 hpf and we know for a fact that CYP activity before and up until this stage is very limited to negligible[29,63]. Furthermore, no internal concentrations of trimethadione and dimethadione were determined. If this had been done, it might be more clear-cut as to whether zebrafish are capable of biotransforming the compounds tested and which of the metabolites are formed by zebrafish embryos. The general concepts in metabolite safety testing of the FDA (but also EMA) state that: "Human metabolites that can raise a safety concern are those formed at greater than 10 percent of total drug-related exposure at steady state. The choice of a level of greater than 10 percent for characterization of drug metabolites reflects consistency with FDA and Environmental Protection Agency guidances" (https://www.fda.gov/downloads/Drugs/.../Guidances/ucm079266.pdf). Indeed analysis of uptake (and consequently internal concentration) of compounds in the zebrafish embryo assay remains one of the most critical factors when interpreting the results. For our study, one could argue that the observed morphological differences for trimethadione and dimethadione might also be due to differences in uptake for both compounds. However, we showed in Chapter 5 that when using dimethadione[64], but starting exposure at 2 hpf, 22.5% of the embryos were malformed at 3 mM. This difference with Chapter 6 is most likely due to a difference in start of exposure. In Chapter 5, we

exposed the embryos earlier (2 hpf) than in Chapter 6 ($5^{1/4}$ hpf). Therefore, we rather detected acute toxicity than teratogenicity. This put aside, we were able to trigger effects from 3 mM dimethadione exposure, indicating uptake of dimethadione is not an issue. Timing of exposure to zebrafish embryos is the critical factor for dimethadione toxicity/teratogenicity.

However, it can be said that selection of trimethadione as the compound of choice might not have been optimal. The rationale behind selecting this compound was because trimethadione was part of another project within our research group, the metabolite (dimethadione) has been used in a previous study (chapter 5)[64] and because optimisation for LCMS was ready to be carried out. Moreover, Weigt *et al*. (2011) state that trimethadione is a proteratogen[2] which is an interesting statement. The (pro-) teratogenic effects of trimethadione are well described in humans[65]. The foetal trimethadione syndrome, which is seen in new-borns after administration of trimethadione during pregnancy, causes malformations such as craniofacial malformations, absence of kidneys and cardiovascular effects, amongst others. However, the effects are mainly due to the teratogenic metabolite, dimethadione as trimethadione is quickly N-demethylated to dimethadione[66]. However, this does not necessarily mean that trimethadione is solely a proteratogen. Studies with fertilised chicken eggs report effects in both tri- and dimethadione exposed eggs, even though the earliest life stages are incapable of biotransforming trimethadione to dimethadione[62]. This indicates a teratogenic effect of trimethadione, as well as its metabolite, dimethadione even though the latter is considered the primary teratogen.

This being said LC-MS analysis of the internal concentrations of trimethadione and dimethadione under MAS and without MAS conditions are needed to gain a complete understanding of exposure, uptake and metabolization and validation of the preincubation approach should be carried out with definite proteratogens to assess the potential of the mZEDTA.

7.10 General conclusion, future perspectives and fields of application

To conclude, a new preincubation strategy for zebrafish developmental toxicity screening of drugs has been developed and seems promising, yet on the short term, bioanalysis of the trimethadione samples is crucial. Subsequently, validation with more tool compounds with definitive pro- or teratogenic actions, as well as non-teratogenic compounds is required. Implementation of the zebrafish embryo assay for regulatory developmental toxicity testing of human pharmaceuticals (e.g. ECVAM) is the ultimate goal but will probably take several years to achieve.

Even though there was a strong focus on pharmaceuticals and the use of the mZEDTA in a pharmaceutical setting, the mZEDTA is not necessarily restricted to drug development. The application of a metabolic activated system for testing of environmental pollutants or testing of complex mixtures

(e.g. waste-water samples) can also be useful. Species such as the zebrafish itself or *Xenopus* are already applied for the examination of water pollutants and complex mixtures and the implementation of a MAS might increase relevancy of the results[54,67]. However, ecotoxicological safety testing is under strict regulations of instances such as REACH that follow OECD validated guidelines. On the other hand, several assays using zebrafish embryos are already validated by OECD (e.g. guideline 236), which might facilitate the transformation of the mZEDTA for ecotoxicology purposes.

Another field of application in which safety testing is required is cosmetics. Concerning cosmetics, it is now mandatory to prove safety of products without using *in vivo* techniques (e.g. cell culture systems). However, as the zebrafish is not considered a laboratory animal, they can be a useful tool for the cosmetic industry. Also food supplements and additives, under supervision by e.g. ECHA can be tested by means of an adapted mZEDTA, showing the broad application field for this alternative model.

7.11 References

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8. Summary

The zebrafish embryo has its value as an alternative model for developmental toxicity/teratogenicity screening. It shows a high concordance with mammalian *in vivo* data and its predictivity is within the same range of other alternatives such as the WEC, EST and MM assay. This, together with the limited cost of housing zebrafish and the fast throughput can have significant financial advantages for pharmaceutical companies. However, the zebrafish assay is also subject to some limitations. For regulatory purposes, there is a clear need for harmonisation of the design, a potential extension of the number of morphological endpoints, which are currently much more limited compared to the mammalian *in vivo* studies (e.g. no skeletal evaluation) and to encompass the lack of biotransformation capacity of the zebrafish embryos themselves. Regarding the latter, a MAS, which is already used in other toxicity assays (e.g. Ames-test with an S9 fraction and FETAX with rat liver microsomes as MAS) could provide a solution. However, currently the use of MAS in the zebrafish embryo assay is very limited due to the inherent toxicity of the MAS for zebrafish embryos.

In this project, we approached this toxicity from several angles by investigating any potential toxicant. As such, temperature, protease inhibitors and the formulation of NADPH played a role in the toxicity. We identified that 0.1M Tris-HCl buffered embryo solution was as a non-toxic medium and the use of NADPH-tetrasodium salt as cofactor, instead of an NADPH regenerating system, was non-toxic as well. Yet, when coincubating this mixture with zebrafish embryos, toxicity remained, indicating that the microsomes themselves are embryotoxic. Compared to the Ames test and FETAX, this is striking, as no toxicity of the MAS in these two tests was reported meaning zebrafish embryos are more sensitive than bacteria and Xenopus embryos/tadpoles.

As a result we shifted from a coincubation strategy towards a preincubation strategy. For this, we preincubated human liver microsomes with NADPH tetrasodium salt and a compound for one hour at 28.5°C. Next we ultracentrifuged the mixture followed by a 1/10 dilution of the supernatant containing a minimal amount of microsomes. Subsequent exposure of zebrafish embryos with this diluted supernatant proved to be non-toxic. This approach will be further validated with several tool compounds in a new project.

9. Samenvatting

Het zebravisembryo wordt gebruikt als alternatief model voor het screenen naar ontwikkelingstoxiciteit van geneesmiddelen. Dit model vertoont een hoge concordantie met *in vivo* data in zoogdieren. Bovendien is de voorspellende waarde van de test vergelijkbaar met andere *in vitro* modellen zoals de WEC, MM en EST. Daarnaast zijn de beperkte kost van het huisvesten en kweken van zebravissen en de korte duur van de test een belangrijk financieel voordeel voor farmaceutische bedrijven. Er zijn echter ook enkele nadelen verbonden aan de ontwikkelingstoxiciteitstest met zebravisembryo's. Aangaande de validatie en aanvaarding van de test door overheidsinstanties is het noodzakelijk dat het protocol en de uitvoering van de test geharmoniseerd wordt zodat een uniform protocol bekomen wordt. Daarnaast is het aantal parameters dat geanalyseerd wordt in de zebravisembyo test beperkter in vergelijking met studies bij zoogdieren (vb. geen skeletale kleuring). Het belangrijkste nadeel is echter het afwezig zijn van een maternale component in het omzetten van de geneesmiddelen. Er zijn namelijk sterke indicaties dat, en zeker in de vroege stadia, de zebravisembryo's niet in staat zijn geneesmiddelen om te zetten in metabolieten. Hierdoor werd geopperd dat implementatie van een exogeen metabool activerend systeem (MAS) soelaas kan bieden. Dit MAS zal namelijk instaan voor de metabolisatie van de geneesmiddelen en werd reeds succesvol gebruikt in andere testen zoals de Ames-test (S9-fractie) en de FETAX (microsomen afkomstig van een rattenlever). Het gebruik van een MAS in de ontwikkelingstoxiciteitstest met zebravisembryo's is echter beperkt omwille van embryotoxiciteit veroorzaakt door dit MAS.

In dit project hebben we verscheidene opties onderzocht die deze toxiciteit kunnen veroorzaken. Als dusdanig werd er toxiciteit waargenomen ten gevolge van de (te hoge) co-incubatietemperatuur, protease inhibitoren en de co-factor NADPH wanneer deze toegediend wordt als een NADPH-regenererend systeem (NRS). Bovendien werd aangetoond dat 0,1M Tris HCL-gebufferd medium het optimale medium is voor co-incubatie doeleinden. Dit medium waarborgt enerzijds namelijk activiteit van de cytochroom P450 (CYP) enzymes, de enzymes in het MAS noodzakelijk voor omzetting van geneesmiddelen en anderzijds is dit medium niet toxisch voor zebravisembryo's. Betreffende de toxiciteit van het NRS werd een oplossing gevonden in de dosering door toediening van een eenmalige dosis NADPH-tetrasodium zout ter vervanging van het NRS. Een eenmalige toediening van dit zout heeft namelijk geen negatief effect op de omzetting van geneesmiddelen en bovendien is dat een niet-toxisch alternatief voor het NRS. Desalniettemin was de embryotoxiciteit nog steeds aanwezig na verscheidene optimalisatiestappen. Dit wijst er waarschijnlijk op dat de microsomen zelf embryotoxisch zijn voor zebravisembryo's. Dit is opmerkelijk, aangezien MAS in zowel de Ames-test

als de FETAX geen toxiciteit uitlokte ten opzichte van respectievelijk de gebruikte bacteriële kolonie en kikkerembryo's.

Ten gevolge hiervan werd er overgegaan van een co-incubatiestrategie naar een pre-incubatiestrategie. Hiertoe werden de microsomen, tezamen met NADPH en een geneesmiddel gedurende één uur geïncubeerd op 28,5°C, waarna dit staal onderworpen werd aan ultracentrifugatie. Vervolgens werd nog een 1/10 verdunning van het supernatans uitgevoerd, waarna deze oplossing gedurende de volledige periode van de organogenese (namelijk vanaf 5.15 uur na fertilisatie tot 96 uur na fertilisatie) kon blootgesteld worden aan de zebravisembryo's en larven. Deze benadering zal in de toekomst verder gevalideerd worden met verscheidene geneesmiddelen.

10. Dankwoord

Het heeft wat voeten in de aarde gehad en uiteindelijk net iets langer dan 4 jaar geduurd, maar ik ben blij dit proefschrift aan iedereen te kunnen laten zien. De tekst neergeschreven in dit werk is echter maar een klein deeltje van een veel grotere ervaring van zowel wetenschappelijke als iets minder wetenschappelijke aard. Gedurende deze 5 jaar heb ik dan ook vele leuke, interessante en grappige mensen mogen ontmoeten en alhoewel werk en privé gescheiden zou moeten blijven ben ik toch blij enkele collega's vrienden te kunnen noemen!

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Verder wil ik Prof. Pieter Annaert en zijn doctoraatsstudenten bedanken om me in te wijden in het opgroeien van en steriel werken met hepatocyten. Ik apprecieer het zeker en vast dat ik met open armen ontvangen werd en dat jullie me op enkele dagen perfect getoond hebben hoe hepatocyten te kweken. Helaas was het geen oplossing voor de toxiciteit!

Furthermore, I would like to thank all members of the ETS taskforce. It was interesting to share different scientific views and listen to the arguments involved to make a thorough decision. I truly hope that one day a uniform protocol will be readily available for all partners. All the best.

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Chloé, the newest member of the team but certainly a good addition! Your enthusiasm and kindness motivates everyone around you. I am sorry, however, that I kicked you so much when we were in the office. But we will blame the small desks. Please take good care of the animals in the pond, now that I am gone. Je vous souhaite un avenir des meilleurs.

Katty, Christel en Gunther, ook jullie ben ik heel dankbaar. Bestellingen regelen, paperassen in orde brengen, altijd bereid zijn te helpen in het labo en de visjes verzorgen zijn maar een aantal zaken in het lijstje. bovendien was het steeds leuk te babbelen met jullie, ik ga deze momenten echt missen.

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2005-2011: Modern languages – Sciences, Royal Athenuem, Ekeren, Belgium

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Optimisation of the co-incubation medium for the mDarT.
At the 43nd Annual Meeting of the European Teratology Society, 30th August-3rd September 2015, Amsterdam, The Netherlands

Temperatures of 32.5°C and above impact zebrafish embryonic development.
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Temperatures of 32.5°C and above impact zebrafish embryonic development.

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Awards

The Elsevier Best Presentation for the presentation:

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At the 46nd Annual Meeting of the European Teratology Society, 10-13th September 2018, Berlin, Germany

The Teddy Edwards' Memorial Award for the presentation:

Temperatures of 32.5°C and above impact zebrafish embryonic development.

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