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Antioxidants reduce reactive oxygen species but not embryotoxicity in the metabolic *Danio rerio* test (mDarT)

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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 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.

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/fetuses 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) metabolite 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. 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 a.o. on exposure duration and varies with the developmental stage at the time of exposure [11]. This has been

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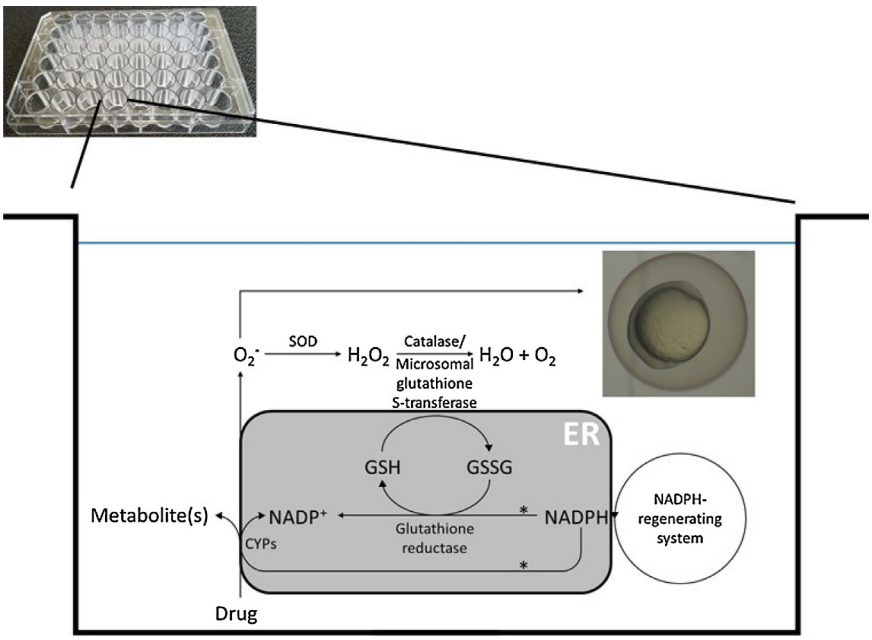

Fig. 1. Simplified scheme of reactive oxygen species formation and reduction in a well of an mDarT co-incubation experiment. GSH: glutathione, GSSG: glutathione disulphide, SOD: superoxide dismutase. Grey compartment represents a microsome, which is a residual vesicle of the endoplasmic reticulum (ER).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

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 1 h or 24 h, 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 1 h (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 1 h [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 Fig. 1), and known to cause cell and tissue damage [18–20], ROS may also play a role in the observed embryotoxicity.

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 (Fig. 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 (Fig. 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] (Fig. 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 1 h 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 1 h exposure (2 hpf until 3 hpf) with exposure of approx. 70 h (2 hpf until 72 hpf). Second, we verified whether pre-incubation with MAS for 1 h 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.

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