Reproductive Toxicology 64 (2016) 50–56

Contents lists available at ScienceDirect

Reproductive Toxicology

journal homepage: www.elsevier.com/locate/reprotox

In vitro CYP1A activity in the zebrafish: temporal but low metabolite levels during organogenesis and lack of gender differences in the adult stage

Moayad Saad[a], Evy Verbueken[a], Casper Pype[a], Christophe Casteleyn[a], Chris Van Ginneken[a], Louis Maes[b], Paul Cos[b], Steven Van Cruchten[a,*]

[a] *Applied Veterinary Morphology, Department of Veterinary Sciences, Faculty of Pharmaceutical, Biomedical and Veterinary Sciences, University of Antwerp, Universiteitsplein 1, B-2610 Wilrijk, Belgium*
[b] *Laboratory of Microbiology, Parasitology and Hygiene, Faculty of Pharmaceutical, Biomedical and Veterinary Sciences, University of Antwerp, Universiteitsplein 1, B-2610 Wilrijk, Belgium*

ARTICLE INFO

Article history:
Received 25 January 2016
Received in revised form 22 March 2016
Accepted 31 March 2016
Available online 1 April 2016

Keywords:
Zebrafish
Embryo
Gender
CYP1A
EROD
Metabolism
Ontogeny
Microsomes

ABSTRACT

The zebrafish (*Danio rerio*) is increasingly used as a screening model for acute, chronic and developmental toxicity. More specifically, the embryo is currently investigated as a replacement of *in vivo* developmental toxicity studies, although its biotransformation capacity remains a point of debate. As the cytochrome P450 1 (CYP1) family plays an important role in the biotransformation of several pollutants and drugs, a quantitative *in vitro* protocol was refined to assess gender- and age-related CYP1A activity in the zebrafish using the ethoxyresorufin-o-deethylase (EROD) assay. Microsomal protein fractions were prepared from livers of adult males and females, ovaries and whole embryo homogenates of different developmental stages. A large biological variation but no gender-related difference in CYP1A activity was observed in adult zebrafish. Embryos showed distinct temporal but low CYP1A activity during organogenesis. These *in vitro* data raise questions on the bioactivation capacity of zebrafish embryos in developmental toxicity studies.

1. Introduction

The zebrafish embryo is gaining a lot of interest as an *in vitro* model to assess the teratogenic liability of drugs and chemicals. Initially intended as a screening tool [1], the focus on the zebrafish embryo is now more directed towards a reduction or even replacement of *in vivo* tests for developmental toxicity [2,3]. The recent acceptance of this model as a validated alternative for acute toxicity testing of chemicals (Test Guideline 236) [4] also triggered its exploration as an alternative for other types of toxicity testing [5,6]. As toxic and pharmacological effects may also be exerted by metabolites of the parent compound, knowledge on the bioactivation potential and overall biotransformation capacity is pivotal for a correct interpretation of toxicity studies in zebrafish. Differences in drug metabolism between zebrafish and man have been reported previously [7], and this has also been shown for other preclinical species, such as rat and dog [8]. However, the biotransformation capacity of zebrafish embryos and possible gender differences in adult zebrafish are still a point of debate and controversy [9,10]. Gender-dependent differences in xenobiotic metabolism have been clearly observed in rats, which justify the use of both genders in toxicokinetic studies. Differences have also been reported between women and men, but inter-individual variability is considered to be more important in humans [11,12]. Therefore, knowledge on the presence or lack of gender-related biotransformation in the zebrafish may help in deciding whether one gender would be representative for the population or whether both genders are required in toxicokinetic studies. Knowledge on the biotransformation capacity of zebrafish embryos is key for the interpretation of developmental toxicity studies, as there is no maternal metabolism of the drug or chemical in contrast to *in vivo* studies in mammals. Zebrafish embryos are directly exposed to the compound via the medium and as such no or negligible bioactivation of the parent compound may lead to false negative results in the case of

* Corresponding author at: University of Antwerp, Antwerp, 2610 Wilrijk, Universiteitsplein 1, Belgium.
E-mail addresses: steven.vancruchten@uantwerpen.be, Steven.VanCruchten@uantwerpen.be (S. Van Cruchten).

http://dx.doi.org/10.1016/j.reprotox.2016.03.049
0890-6238/

proteratogens, which impacts human risk assessment. The ultimate proof of lack or presence of bioactivation in the zebrafish embryo would be the absence or presence of malformations in embryos exposed to proteratogens. This has been done before [13]. However, the data are difficult to interpret, as one would need to be sure that only the metabolite (s) is/are teratogenic for the zebrafish embryo and not the parent compound itself. Additionally, other factors than bioactivation, such as absorption, distribution and excretion of the compound may complicate the results of a developmental toxicity study in the zebrafish. Therefore, we decided to first investigate the intrinsic biotransformation potential of zebrafish *in vitro*, *i.e.* by using microsomes of whole embryo homogenates and adult livers. These microsomes, which are subcellular fractions of endoplasmic reticulum obtained by (ultra)centrifugation steps, contain highly concentrated cytochrome P450 enzymes (CYPs) [14].

CYPs, and in particular the CYP1, CYP2 and CYP3 families, are the most important groups of enzymes for bioactivation of xenobiotics [15]. For the zebrafish, the genetic features and synteny are well characterized for the different CYP isoforms, as is their expression in different organs. However, CYP activity data are either scarce or conflicting [7]. The CYP1 family is of a particular interest as it has a broad spectrum of substrates, including drugs and environmental pollutants [16]. The ethoxyresorufin-o-deethylase (EROD) assay is one of the most commonly used tests to assess CYP1 activity in humans and fish, including zebrafish [17,18], especially for CYP1A1 and CYP1A, respectively [19,20]. The high affinity of zebrafish CYP1A for ethoxyresorufin (ER) has been clearly demonstrated, as EROD activity was much lower in CYP1A morpholinos than in CYP1B1 morpholinos [21,22]. However, differences in study design, including applied substrate concentration, gender, developmental stage, incubation temperature, *etc.* make the interpretation of the available EROD data for adult zebrafish and embryos very difficult (Table 1).

The aims of the present study were to set-up a standardized *in vitro* protocol to assess CYP1A activity in zebrafish and to apply this assay to different developmental stages during organogenesis to determine the biotransformation potential of zebrafish embryos during their critical window for teratogens. In addition, possible gender differences were assessed in adult zebrafish as this may explain discrepancies between studies and as such also help deciding whether both genders should be used in toxicokinetic studies or not.

2. Material and methods

2.1. Chemicals

Phenylmethanesulfonyl fluoride (PMSF), 1,10-phenanthroline (PHEN), Ethylenediaminetetraacetic acid (EDTA), dimethyl sulfoxide (DMSO), Oil Red O, ER and resorufin (RS) were purchased from Sigma–Aldrich (St. Louis, MO, USA). KH_2PO_4/K_2HPO_4 buffer was purchased from BD Gentest (Woburn, MA, USA). NADPH regenerating system solution A and B were obtained from Corning (Woburn, MA, USA). Total protein content was determined using the Pierce bicinchoninic acid assay (BCA Assay; Pierce Chemical, Rockford, IL, USA). This company also delivered the Halt Protease Inhibitor Cocktail®. Stock solutions of RS and ER were made by dissolving 1 mg in 50 ml and 5 ml DMSO, respectively. Aliquots were made and stored in dark reaction tubes (Greiner Bio-One GmbH, Frickenhausen, Germany) at −80 °C. The absorbance was measured at 572 nm for RS and 482 nm for ER after diluting the stock solution 1:3 and 1:100 in 100 mM KH_2PO_4/K_2HPO_4 buffer. The exact concentrations were calculated using the extinction coefficient (ε RS = 73.2 M^{-1} CM^{-1}, ε ER = 73.2 M^{-1} CM^{-1}) [29,30].

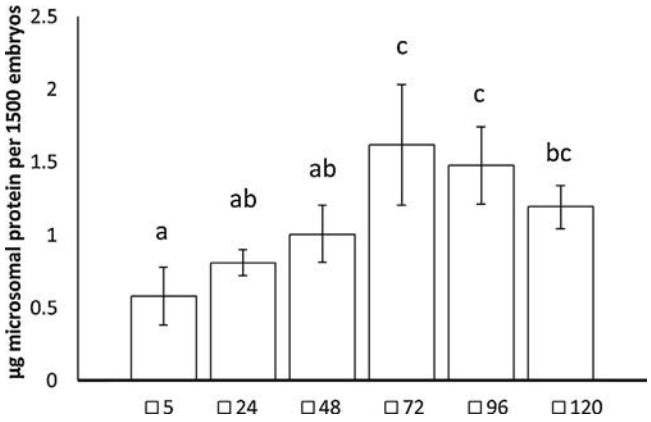

Fig. 1. Microsomal protein yield of zebrafish embryos at different developmental stages (5, 24, 48, 72, 96 and 120 hpf). Bars represent the yield at each stage (mean ± SD, n = 5 batches of 1500 embryos). Significant differences ($p \leq 0.05$) between developmental stages are indicated by different letters.

2.2. Animals and breeding

Adult (one to two years old) zebrafish (*Danio rerio*, in house wild type AB zebrafish line) were kept in glass aquaria of 60 l at a density of 1 fish/L, using a filtered system and day-night rhythm of 14/10 h. The water parameters were 28 ± 1 °C, 500 ± 40 µS cm^{-1} and 7.5 ± 0.3 of temperature, conductivity and pH. The water was renewed at least once in a fortnight to keep the levels of ammonia, nitrite and nitrate below the detection limits. Fish were fed three times daily: twice daily with thawed food (*Artemia nauplii*, *Daphnia* or Chironomidae larvae) and once daily with granulated food (Biogran medium; Prodac International, Cittadella, Italy). For embryo collection, the fish were put in spawning tanks before the light was turned on. Embryos were collected 45 min after the start of spawning. Feces and coagulated eggs were removed and the embryos were gently washed using freshly prepared embryo solution, *i.e.* Instant Ocean® Sea Salt (Blacksburg, VA, USA) and sodium bicarbonate (VWR, Leuven, Belgium) dissolved in reverse osmosis water (conductivity 500 µS.cm^{-1}; pH 7.5). The embryos were kept in embryo solution under the same environmental conditions of light and temperature as for the adults. The embryo solution was renewed daily and dead embryos were removed. When the embryos reached the desired developmental stage, they were frozen in liquid nitrogen and stored at −80 °C to be used for the preparation of the embryo microsomal protein.

2.3. Tissue sampling

For the preparation of adult liver microsomes, each batch consisted of 10 females or 10 males. In total, five batches of each gender were prepared. Animals were fasted for 48 h before they were euthanized by decapitation followed by a rapid destruction of the brains (Recommendation 2007/526/EC, Species Specific Section, Humane Killing Fish, p89). Livers were rinsed during the dissection process with pre-cooled washing buffer (100 mM KCl, 1 mM EDTA and 10 mM KH_2PO_4/K_2HPO_4 buffer at pH 7.4) to remove the remaining blood and avoid bile contamination. Additionally, both ovaries of 20 adult zebrafish were collected for microsomal protein preparation. For the embryo microsomal protein, five batches of embryos were collected for each of the six different developmental stages, *i.e.* 5, 24, 48, 72, 96 and 120 hpf (hours post-fertilization). In order to have a sufficient yield of microsomal protein, each batch consisted of at least 1500 embryos. The livers, ovaries and whole embryos were stored at −80 °C until further use. The Ethical Committee of Animal Experimentation from the Uni-