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Paracetamol-induced liver injury modelled in *Xenopus laevis* embryos

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ABSTRACT

Introduction: Failure to predict drug-induced liver injury (DILI) remains a major contributing factor to lead compound drop-out during drug development. *Xenopus* embryos are amenable for early stage medium throughput small molecule screens and so have the potential to be used in pre-clinical screens. To begin to assess the usefulness and limitations of *Xenopus* embryos for safety assessment in the early phases of drug development, paracetamol was used as a model hepatotoxin. Paracetamol overdose is associated with acute liver injury. In mammals, the main mechanism of paracetamol-induced acute liver injury is an increased amount of the reactive metabolite *N*-acetyl-p-benzoquinone imine (NAPQI) combined with a reduction of free glutathione (GSH). Humans that have taken an overdose of paracetamol are often treated with *N*-acetyl cysteine (NAC).

Method: Xenopus laevis embryos were treated with up to 5 mM paracetamol from stage 38 to stage 45 during development, when the liver is functional. The presence of paracetamol-induced liver injury was assessed by: (1) microRNA-122 (miR-122) expression (a hepatic marker), (2) free GSH concentration (a marker of oxidative stress) and (3) NAC antioxidant intervention.

Results: The amount of free GSH decreased significantly in embryos exposed to increasing paracetamol concentration. In embryos exposed to 5 mM paracetamol, 22.57 ± 4.25 nmol/mg GSH was detected compared to 47.11 ± 7.31 nmol/mg untreated embryos (mean ± SEM). In tail tissue, miRNA-122 expression increased 6.3-fold with 3 mM paracetamol concentration treatment compared to untreated embryos. NAC treatment altered the free GSH decline for embryos treated with up to 5 mM. Embryos exposed to 1 mM paracetamol and then exposed to 0.5 mM NAC 24 h prior to harvest, had a significantly higher amount of GSH compared to embryos that were only exposed to 1 mM paracetamol (mean ± SEM; 97.1 ± 9.6 nmol/mg and 54.5 ± 6.6 nmol/mg respectively).

Conclusion: Xenopus laevis embryos exhibit similar characteristics of paracetamol-induced liver injury observed in mammalian models. These data indicate that the *Xenopus* embryo could be a useful *in vivo* model to assess DILI and aid lead compound prioritisation during the early phase of drug development, in combination with pre-clinical *in vitro* studies. Consequently, the *Xenopus* embryo could contribute to the reduction principle as defined by the National Centre for the Replacement, Refinement and Reduction of Animals in Research.

1. Introduction

Paracetamol is a frequently used antipyretic and analgesic drug that is safe at the therapeutic dose, which is 4 g/day. However, it exhibits dose-dependent hepatotoxicity and severe liver injury can occur when a single dose of 10–15 g of paracetamol is taken (Dart et al., 2006; Jaeschke, 2015). Paracetamol contributes to 80% of the liver failure cases associated with drugs (Larson et al., 2005). It is the single largest cause of acute liver failure in the US and the UK and the second most common reason for a liver transplant (Ostapowicz et al., 2002). In the UK, it is estimated approximately 90,000 patients present with paracetamol overdose a year, resulting in 50,000 hospital admissions and 150–250 deaths (Bateman et al., 2014; Wong et al., 2014; Hawton et al., 2013). The paracetamol reactive metabolite that is responsible for paracetamol-induced liver injury is *N*-acetyl-p benzoquinone imine (NAPQI). When a paracetamol overdose has been taken, the supply of cellular glutathione (GSH) is saturated and therefore less NAPQI is neutralised. In hepatocytes, NAPQI can form protein adducts with intracellular proteins, which can lead to mitochondrial dysfunction, nuclear DNA fragmentation, oxidative stress, hepatocyte death and subsequent acute liver failure, and potentially patient death (Prescott, 1980; Jaeschke et al., 2012). The major cell death pathway is necrotic, although some apoptotic cell death can also occur.

Xenopus laevis (the African clawed frog) can produce approximately

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1000 embryos/day with hormonal stimulation. Following *in vitro* fertilisation, the embryos rapidly develop *ex utero* and are amenable for medium throughput small molecule screens. As part of these screens, it was noted that *Xenopus* embryos could highlight compound toxicity (Tomlinson et al., 2012, 2005, 2009). Consequently, as well as being useful to identify new drugs, *Xenopus* embryos may also represent a model for early phase pre-clinical drug toxicity studies during drug development. Previously, we have successfully used this model to help assess nanotoxicity for biomedically-relevant nanomaterials (Webster et al., 2016).

The *Xenopus* embryo shares similar advantages to another non-mammalian animal model, the zebrafish (*Danio rerio*) (Wheeler and Brändli, 2009). They are both easier to house and less expensive than mammalian animal models. However, unlike in zebrafish, not much work has been done to investigate the use of *Xenopus* embryos for the prediction of drug-induced toxicity.

Drug-induced liver injury (DILI) was investigated in order to begin to characterise the use of *Xenopus* embryos as a predictive tool for drug-induced toxicity. Paracetamol was used as the model hepatotoxin. Paracetamol-induced liver injury is dose-dependent. Therefore it was hypothesised that the *Xenopus laevis* embryos would exhibit indications of paracetamol-induced liver injury that correlated with paracetamol dose exposure. Increasing paracetamol concentration exposure to the *Xenopus* embryos, correlated with an increased expression of the liver-specific biomarker miR-122 in tissue that does not include the liver, and a depletion of free GSH.

2. Methods

All experiments were performed in compliance with the relevant laws and institutional guidelines at the University of East Anglia. This research has been approved by the local ethical review committee according to UK Home Office regulations. *Xenopus* embryo stages were identified according to Nieuwkoop and Faber (1994).

Xenopus laevis embryos develop at a rate that is proportional to the temperature at which they are incubated. Consequently, unlike the zebrafish, the age of a *Xenopus* embryo cannot be given in hours post fertilisation (hpf), instead, the age can be identified using the developmental stages set out by Nieuwkoop and Faber (these can also be found on the NIH-funded website xenbase.org) (Nieuwkoop and Faber, 1994). At 25 °C, a *Xenopus* embryo can develop into an adult in 12 months, this is the temperature that elicits the fastest development rate. At < 12 °C the embryos will die however, between 12 °C and 25 °C, the embryos will develop at a rate proportional to the temperature. An incubation temperature of 23 °C was used for all of the following experiments.

2.1. Embryo fixing

Once the embryos reached their desired stage they were fixed using MEMFA (3.7% (v/v) formaldehyde, 1X MEM salts (0.1 M MOPS, 2 mM EGTA, 1 mM MgSO4, pH7.4) made up with dH_2O. They were then dehydrated in 100% ethanol (EtOH) and stored at −20 °C.

2.2. RNA isolation and cDNA synthesis

After incubation until the stage required, 10 embryos were frozen in liquid nitrogen for at least 2 h. mRNA was isolated using the trizol method, miRNA was isolated using the miRCURY™ RNA Isolation (for tissue) kit according to manufacturer's instructions. For miRNA, cDNA was generated using the Universal cDNA synthesis kit II (Exiqon).

2.3. qRT-PCR

The reaction was performed in MicroAmp optical 96 well plate (Applied Biosystems)., miRCURY™ LNA PCR Primer mix (Exiqon) or 10 μM MRP2 primer and SYBR® Green PCR Master Mix (Applied Biosystems). A 7500 real-time PCR instrument (Applied Biosystems) was used under the following conditions. Samples were plated in triplicates (technical replicates). Samples derived from embryos produced from different mothers were the biological replicates.

2.4. Wholemount in situ hybridisation (WISH)

DIG labelled probes were synthetised with a Digoxigenin (DIG) labeled UTPs (Roche) and purified using Illustra MicroSpin G-50 Columns (GE healthcare life sciences) according to manufacturer's instructions. Micro-RNA probes were obtained from Exiqon. WISH was carried out as previously described (Ahmed et al., 2015; Harrison et al., 2004)

2.5. Paracetamol treatment

Paracetamol (Sigma, UK) was dissolved in 0.1X MMR alone. Embryo age was determined according to Nieuwkoop and Faber (1994). For 1 biological replicate (n = 1), we used 7 embryos for each drug concentration. Consequently 5 biological replicates (n = 5) used a total of 35 embryos. 1 biological replicate is defined as using 1 adult frog mother. In other words, a result generated from embryos that have the same mother, was only counted as 1 biological replicate.

2.6. The preparation of samples for the measurement of miR-122 expression using qRT-PCR

Stage 38 embryos were treated with paracetamol as described above until stage 45. At stage 45, the embryos put into 0.5 mg/mL tricaine (0.1X MMR) and incubated at 23 °C for 1 h. Embryos were dissected into tail and gut tissue using a simple razor blade. The dissection technique was confirmed by analyzing the expression of the liver-specific marker AMBP. AMBP was expressed in the gut tissue dissected but not in the tail tissue.

2.7. N-acetyl cysteine (NAC) and paracetamol

2.7.1. Pre-incubation

Stage 38 embryos were exposed to a final concentration of 0.5 mM NAC at 23 °C for 2 h prior to paracetamol. This was incubated for a further 70 h, until the embryos were stage 45 at 23 °C. The embryos were processed with the GSH assay method.

2.7.2. Concurrent treatment

Stage 38 embryos were exposed to a final concentration of 0.5 mM NAC and a paracetamol concentration within the range 0–5 mM paracetamol at 23 °C for 72 h until the embryos were stage 45. The embryos were processed with the GSH assay method.

2.7.3. 24 h NAC treatment prior to harvest

Stage 38 embryos were exposed to paracetamol within the range 0–5 mM at 23 °C for 48 h. At 48 h we added NAC to a final concentration of 0.5 mM and this was further incubated for 24 h at 23 °C until the embryos were stage 45.

All the embryos were processed with the GSH assay method at stage 45.

2.8. Measuring free GSH

2.8.1. Sample preparation

At the end of the incubation period, embryos that were treated with the same conditions were pooled. As much as possible, the incubation solution was removed and the embryos were homogenized. The amount of free GSH was measured in the supernatant using a GloMax® Explorer System spectrophotometer (Promega) and quantified using the Bradford assay.

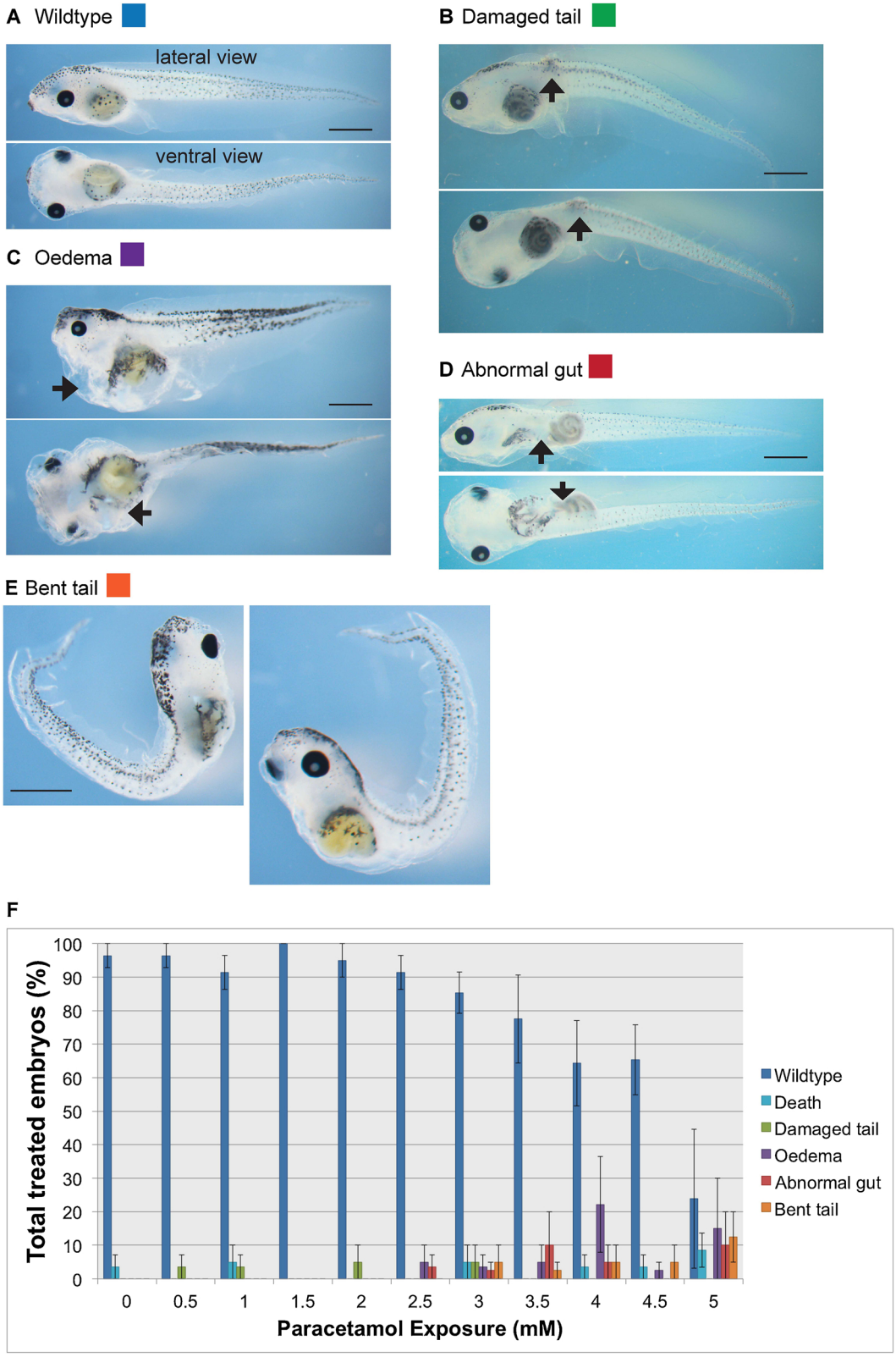

Fig. 1. Paracetamol exposure dose-response phenotype.
Xenopus laevis embryos were exposed to a paracetamol concentration in the range 0–5 mM (n = 4). The embryos were exposed to paracetamol from stage 38 and harvested at stage 45. Phenotypes include wildtype (A), damaged tail (B), oedema (C), abnormal gut (D) and bent tail (E). The surviving embryos at stage 45 were photographed and the different phenotypes found for each paracetamol concentration were calculated as a percentage of total embryos tested (F). Where possible, the embryos were photographed to show the lateral view and the ventral view. Scale bar represents 1 mm.

2.9. Statistical analysis

Statistical tests were carried out using GraphPad Prism 6.0 software. An ordinary one-way ANOVA was used for investigating the difference of the treated groups compared to the untreated embryos and Mann-Whitney non-parametric tests were used to compare unpaired treatment groups.

3. Results

3.1. Paracetamol exposure response in Xenopus laevis embryos

Stage 38 *Xenopus laevis* embryos were exposed to paracetamol that was dissolved into the embryo incubation media. After a 72 h incubation period, the resulting stage 45 embryos showed a variety of phenotypes (Fig. 1). Untreated stage 45 *Xenopus* embryos were termed "wildtype". In the treatment group, the wildtype phenotype was less common with increasing paracetamol concentration. Of the embryos that were exposed to the *Xenopus* media only, 96% displayed a wildtype phenotype, which decreased to 24% for the embryos exposed to 5 mM paracetamol.

Phenotypes that deviated from the norm included damaged tail, oedema, abnormal gut and bent tail **(**Fig. 1A–E**)**. The incidence of all phenotypes that are different to the wildtype was larger with increasing paracetamol concentration from 4% to 46%, *Xenopus* media only to 5 mM paracetamol exposure respectively **(**Fig. 1F**)**. Oedema typically occurred around the gastro-intestinal region of the embryo **(**Fig. 1C**)**. The percentage of stage 38 embryos that were exposed to 5 mM paracetamol and did not survive to the harvest age of stage 45 was 9% compared to the 4% incidence for the embryos exposed to *Xenopus* media only.

3.2. Free GSH content in paracetamol treated embryos

In humans, the generation of the reactive metabolite, NAPQI, and the reduction of the neutralising agent, GSH, mediate paracetamol-induced liver injury. Consequently, the amount of free GSH can indicate the metabolism pathway of paracetamol and be an indirect measurement of paracetamol-induced liver injury. Existing *in vivo* or *in vitro* assays that imitate human paracetamol overdose and the associated acute liver failure, show a reduction of free GSH in the assay system (Howell et al., 2014; Shenton et al., 2004; Goldring et al., 2004; Vliegenthart et al., 2017).

Embryos at stage 38 were treated with a paracetamol concentration within the range 0–5 mM, harvested at stage 45, and processed to measure the amount of free GSH (n = 10) (Fig. 2). The amount of free GSH decreased from 47 nmol/mg to 22 nmol/mg with increasing concentration of paracetamol (0–5 mM). Embryos treated with a paracetamol concentration within the range 3–5 mM had statistically significantly less free GSH compared to untreated embryos (5 mM paracetamol exposure mean ± SEM was 22.57 ± 4.25 nmol/mg compared to untreated mean ± SEM of 47.11 ± 7.31 nmol/mg). *Xenopus laevis* embryos that are incubated at 23 °C take 72 h to develop from stage 38 to stage 45. In order to explore the time-dependency of the decline in free GSH, stage 38 embryos were treated with the same paracetamol concentration range (0–5 mM) and harvested after 24 h, at stage 41 (Fig. 2B). There was no significant change in the relationship between paracetamol treatment and amount of free GSH when the embryos are treated for 24 h.

3.3. miR-122 is a marker for the liver in Xenopus embryos

The location of the liver is not always obvious in the *Xenopus laevis* embryo, it is < 0.5 mm at its widest diameter, and the colour of it is not notably different to the intestines. Thus, a probe was used to identify the liver using a wholemount *in situ* hybridisation (WISH) protocol. Alpha-1-microglobulin/bikunin precursor (AMBP) is a protein specifically expressed in the *Xenopus* liver (Zorn and Mason, 2001) and so it was used as a positive control for the WISH. Stage 38 and stage 45 embryos expressed AMBP in the presumed location of the liver **(**Fig. 3A**)** (n = 10).

The expression of miR-122 in the *Xenopus* embryos was investigated using the WISH assay (Ahmed et al., 2015) (Fig. 3B) (n = 10). MiR-122 expression is specific to the liver in humans as well as rodent and zebrafish animal models (Starkey Lewis et al., 2011; Vliegenthart et al., 2015; Antoine et al., 2013). Elevated miR-122 in the circulation can therefore serve as a biomarker for DILI (Wang et al., 2009). The miR-122 WISH probe has been extensively characterised in *Xenopus* previously (Ahmed et al., 2015). The WISH for miR-122 stained the same area as AMBP in stage 38 and stage 45 embryos (Fig. 3). Therefore, it was concluded that the miR-122 expression in *Xenopus laevis* is specific to the liver tissue, which is very similar to the expression seen in rodent, zebrafish and humans.

3.4. The expression of miR-122 in the different tissues of embryos treated with paracetamol

Stage 38 embryos were exposed to a paracetamol concentration within the range 0–5 mM, harvested at stage 45 and then processed to measure the expression of miR-122 using qRT-PCR. Embryos were dissected to obtain gut tissue that included the liver and tail tissue to represent the blood. The tail contains a good vasculature and the liver tissue is not present in this body compartment (Supplementary Fig. 1**)**.

The miR-122 expression levels were normalised to a miRNA (miR-103), which has been shown to not be affected by paracetamol treatment in rodents (Wang et al., 2013). Similarly, the expression of miR-103 is not affected by paracetamol in *Xenopus* laevis (Supplementary Fig. 2). The miR-122 expression was also normalised to untreated (*Xenopus* media only) embryos (Fig. 4).

The expression of miR-122 increased in the tail tissue with increasing paracetamol concentration. In untreated wildtype embryos, the miR-122 expression in the tail was minimal. miR-122 expression in the tail of embryos treated with 3 mM and 4 mM paracetamol was significantly different compared with miR-122 expression in the gut tissue from the same paracetomol-treated embryos (Fig. 4). Embryos treated with the 3 mM paracetamol concentration in particular, had a 6.3-fold (plotted as 0.8 on the logarithmic y-axis scale) increase in miR-122 expression in the tail, compared with the tail tissue of untreated embryos. In the gut tissue, the expression of miR-122 appears to be slightly reduced in treated embryos compared with untreated embryos, but miR-122 expression does not change in relation to the paracetamol concentration, it remains level (Fig. 4).

3.5. Treatment with acetyl-meta-aminophenol (AMAP)

AMAP is supposedly the non-toxic regioisomer version of APAP, but this appears to be species-dependent (Hadi et al., 2013). The *Xenopus* response to AMAP was explored by looking at the effect of GSH depletion, which was determined by measuring the amount of free GSH in embryos from the same mother, treated with either AMAP or APAP in the same concentration range (0–5 mM, n = 3). The embryos treated with AMAP produced a negative correlation: as the concentration of AMAP increased, the amount of free GSH detected decreased (Fig. 5). The amount of GSH between embryos treated with the same concentration of either AMAP or APAP was not significantly different.

3.6. Xenopus incubation with the human clinical treatment for paracetamol overdose

In the clinic, patients are administered *N*-acetyl cysteine (NAC) to prevent severe paracetamol-induced liver injury. The NAC replenishes GSH stores, scavenges ROS in mitochondria and enhances the

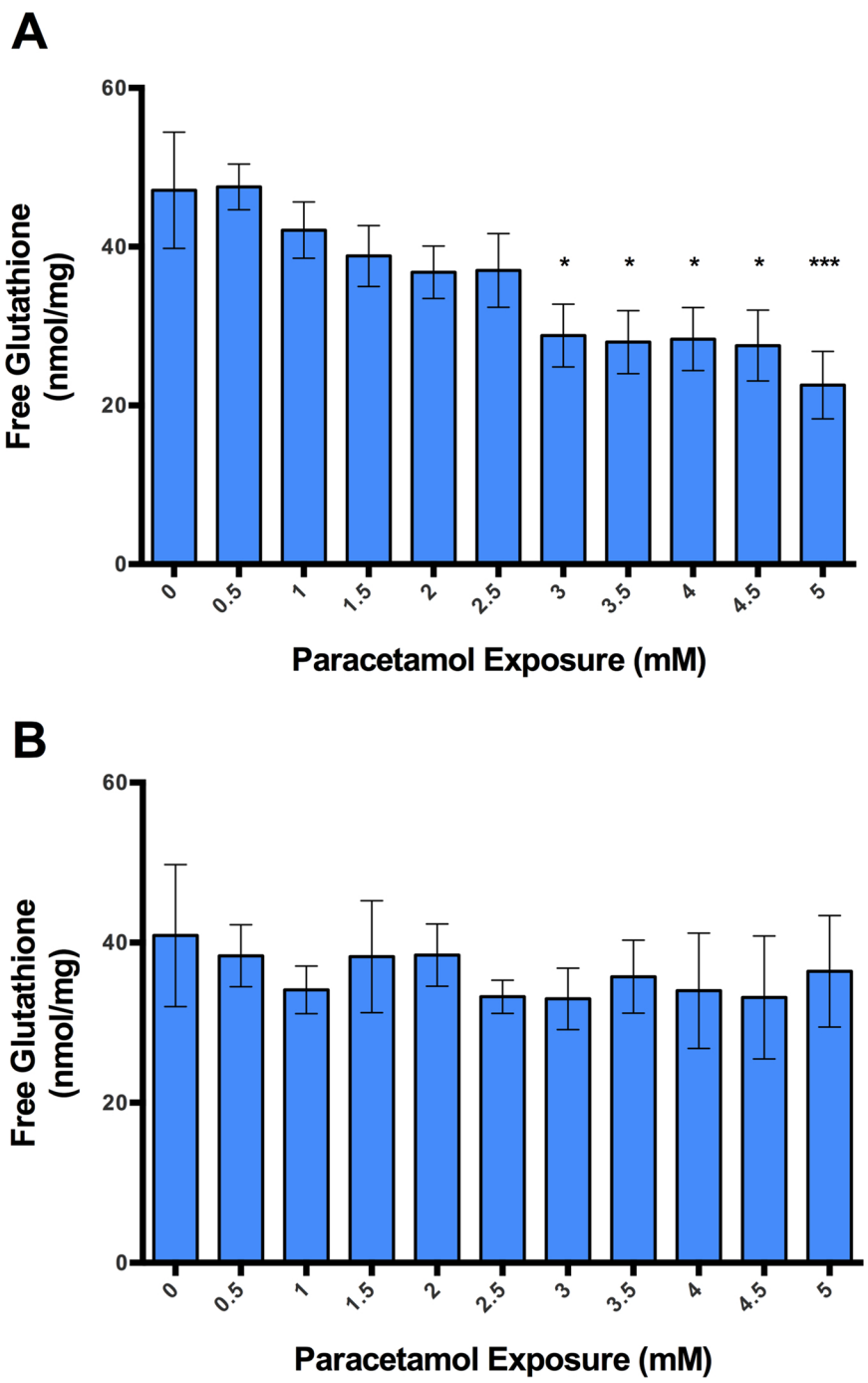

Fig. 2. The amount of free GSH inside *Xenopus* embryos treated with paracetamol.
Xenopus laevis embryos were exposed to 0–5 mM paracetamol from the age of stage 38, and harvested at stage 45 (A, n = 10) or at stage 41 (B, n = 3). The amount of free GSH (nmol/mL) was measured inside the embryos and normalised to the amount of protein (mg/mL). An ordinary one-way ANOVA compared each exposure group to the group exposed to *Xenopus* media alone (0 mM). For the embryos exposed until stage 45 (A), the amount of free GSH for 3, 3.5, 4 and 4.5 mM paracetamol exposure groups was statistically significant compared to the group exposed to *Xenopus* media only ($P < 0.05$). The 5 mM paracetamol exposure group was also statistically significantly different ($P < 0.01$). There was no significant difference between treated and untreated embryos that were harvested at stage 41 (B).

sulphation APAP metabolic pathway. The NAC therapeutic effect is more useful at preventing liver injury if it is taken less than 8 h after the APAP overdose (Waring, 2012; Smilkstein et al., 1988).

Xenopus embryos were co-incubated with 0.5 mM NAC and a concentration within the 0–5 mM APAP range. Initially, NAC and APAP were co-incubated in 2 conditions: (Dart et al., 2006) incubation of the stage 38 embryos with 0.5 mM NAC for 2 h and then the APAP concentration was added to the media and (Jaeschke, 2015) concurrent 0.5 mM NAC and APAP (n = 10) **(**Fig. 6**A)**. The embryos were harvested at stage 45 and compared to embryos treated with APAP alone. The free GSH concentration was measured and normalised to the untreated embryos for each condition, which are denoted as a percentage of the 0 mM treatment group. Embryos treated with NAC alone did not have a significantly different amount of free GSH to the untreated wildtype embryo. Embryos that were pre-incubated with NAC before the APAP addition, had a decrease in free GSH with increasing APAP concentration, however the gradient was smaller in comparison to their counterparts that were treated with APAP only (Fig. 6A). The concurrent NAC and APAP co-incubation also had a negative correlation between free GSH and APAP concentration, but this gradient was (on average), smaller than the pre-incubation treatment. Both methods showed a trend where NAC reduced the effect of APAP on GSH, but this was not statistically significant.

Next, 0.5 mM NAC was added to APAP-treated embryos 24 h, prior to their harvest at stage 45 (n = 5) **(**Fig. 6**B)**. The free GSH measured in the embryos treated with NAC was (on average), higher than the counterpart embryos treated with the same APAP concentration compared with APAP alone. However, the amount of free GSH measured in NAC-treated embryos did decrease with increasing APAP concentration, though significantly less than in embryos treated with APAP alone.

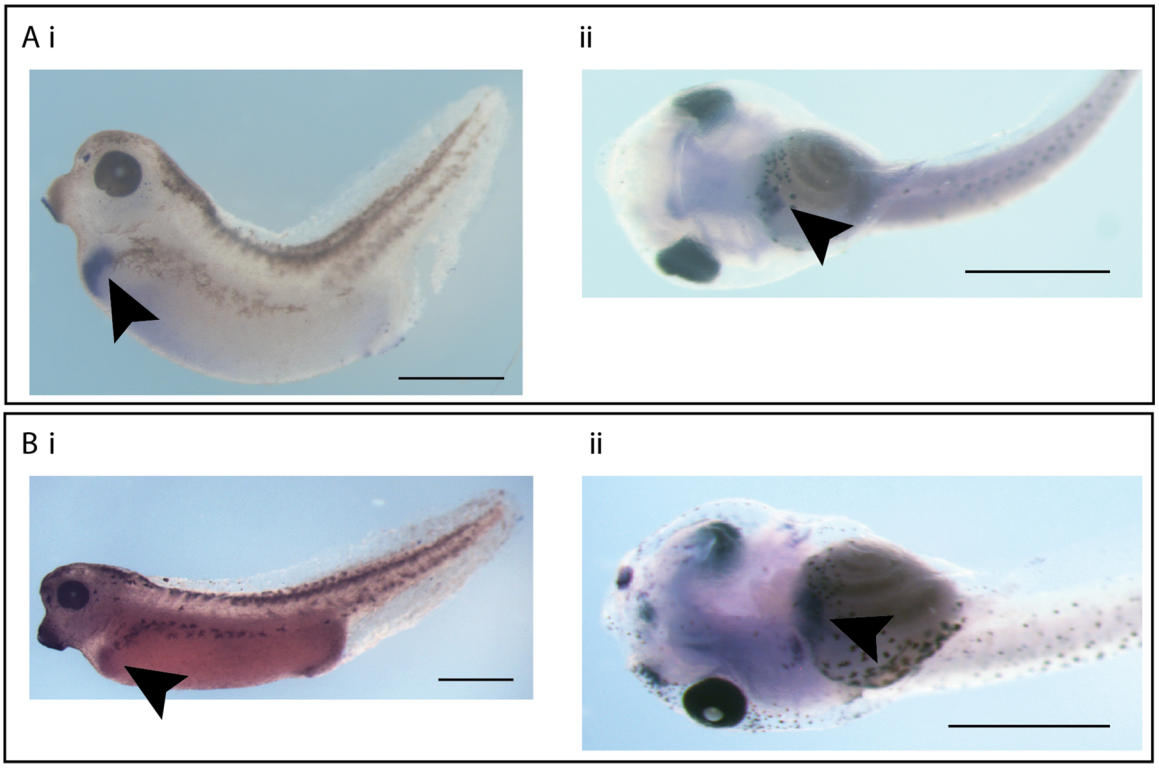

Fig. 3. Wholemount *in situ* hybridisation (WISH) for liver markers.
WISH assay for Alpha-1-Microgobulin/Bikunin Precursor (AMBP) (A) and miR-122 (B) at stage 38 (Ai and Bi) and stage 45 (Aii and Bii). The stage 38 embryos are shown in lateral view and the stage 45 embryos are shown ventrally. The specific purple WISH stain is indicated with an arrow. These embryos are representatives of the typical expression patterns seen (n = 10). Scale bar represents 1 mm.

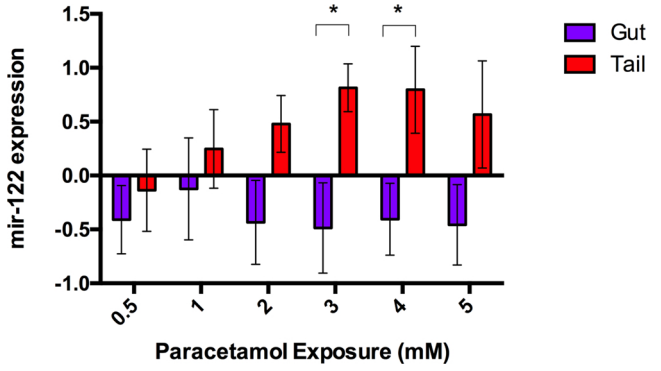

Fig. 4. Expression of miR-122 in paracetamol-treated embryos.
Stage 38 embryos were exposed to paracetamol (0–5 mM) and harvested at stage 45. The expression of miR-122 in the gut (purple) and tail (red) was measured using qRT-PCR (log (fold change ± SEM)) and normalised to embryos exposed to *Xenopus* media only (0 mM). The statistically significant difference of miR-122 expression between tissues from embryos treated with the same paracetamol concentration was measured using the Mann-Whitney test ($P < 0.05$) (n = 5).

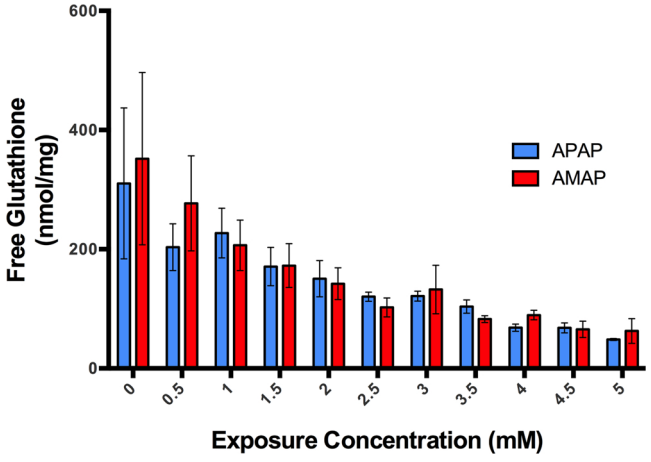

Fig. 5. The amount of free GSH inside *Xenopus* embryos treated with paracetamol (APAP) or *N*-acetyl-meta-aminophenol (AMAP).
Xenopus laevis embryos were exposed to up to 5 mM paracetamol (APAP) or up to 5 mM *N*-acetyl-meta-aminophenol (AMAP) from the age of stage 38 and harvested at stage 45 (n = 3). The amount of free GSH (nmol/mL) was measured inside the embryos and normalised to the amount of protein (mg/mL). There was no significant difference between embryos treated with the same concentration of APAP and AMAP.

4. Discussion

To our knowledge, the *Xenopus laevis* animal model has not been thoroughly assessed as a model to predict drug-induced toxicity. To begin to investigate the usefulness and limitations of *Xenopus laevis* embryos in this area, this research focused on the response to one known hepatotoxic drug, paracetamol. The results generated here suggest that *Xenopus* could be a suitable model to predict DILI.

The *Xenopus* liver has the same cell types as found in humans including hepatocytes, stellate cells, Kupffer cells and sinusoidal endothelial cells (Blitz et al., 2006). Similar to vertebrates, the liver is derived from the endoderm of the future gut tube, close to where the stomach and duodenum will meet (Nieuwkoop and Faber, 1994). By tadpole stage 37–39, the *Xenopus* liver is a sac-like structure, with thick walls that fold inwards and fills the liver cavity with hepatocytes. Also at this time, the liver and biliary ductal systems are developing and the gall bladder is a thin-walled sac structure (Blitz et al., 2006). At late stage development (stage 38–45) the *Xenopus* heart, liver and kidney

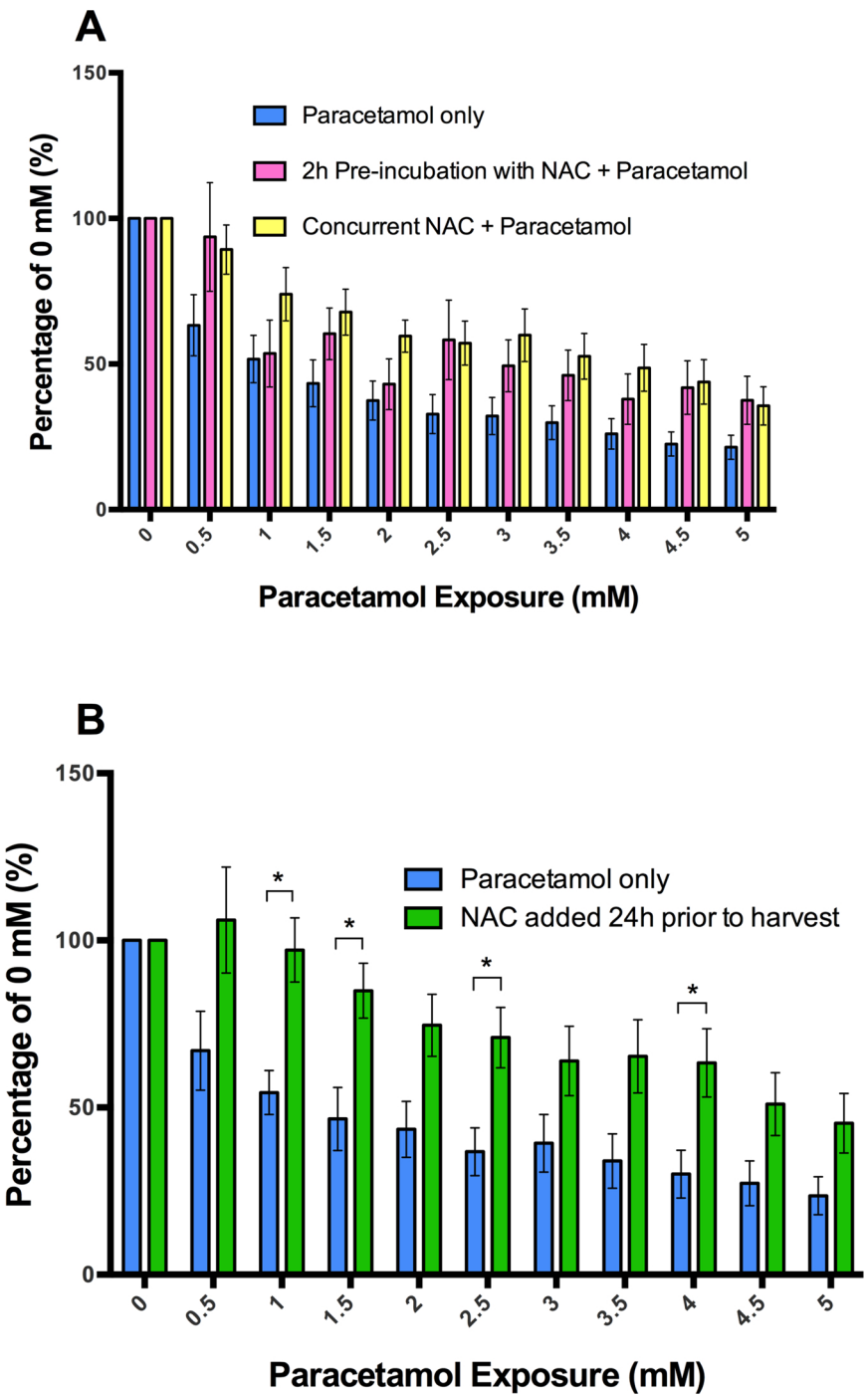

Fig. 6. Free GSH in embryos treated with paracetamol and *N*-acetyl cysteine (NAC).
Stage 38 embryos were exposed to paracetamol (up to 5 mM) for 72 h with or without the addition of 0.5 mM NAC, they were harvested at stage 45. For the initial experiments (A) there were 3 conditions: exposure to paracetamol alone (blue), 2 h incubation with NAC prior to the addition of paracetamol (pink) and concurrent treatment with NAC and paracetamol (yellow) (n = 10). The embryos were also exposed to APAP from stage 38 and then with NAC for 24 h prior to harvest (green) at stage 45, a 72 h incubation period, or with paracetamol alone (blue) (B) (n = 5). The amount of free GSH inside the embryos was measured and normalised to the amount of protein. This value was then normalised to the embryos exposed to *Xenopus* media alone (0 mM). The amount of free GSH measured was calculated as a percentage of the 0 mM paracetamol exposure group (± SEM). A multiple comparison 2 way ANOVA was performed to measure the statistical significance between groups of them same paracetamol exposure, with and without additional NAC exposure ($P < 0.05$).

are functional and adding compounds at this stage would assess drug-induced toxicity. Of the key enzymes involved in paracetamol metabolism in humans, the *Xenopus* enzymes of the same name are also expressed at stage 38 and stage 45. Including the cytochrome P450 isoform CYP2E1, glutathione S-transferase (GST) isoforms P1, M1 and T1, sulphotransferase (SULT) isoforms 1A1 and 2A1 and uridine 5′-diphospho-glucuronosyltransferase (UGT) 1A6 and 1A1 (Supplementary Fig. 3).

It was hypothesised that toxicity assessment using the *Xenopus* embryos in combination with existing *in vitro* pharmacological cytotoxicity profiling and mathematical modelling can provide early *in vivo* testing without the need for extensive early mammalian testing. This will be consistent with the reduction ideology of the National Centre for the Replacement, Refinement and Reduction of Animals in research (NC3R). Experiments using *Xenopus* could help prioritise the lead compounds and provide more information than *in vitro* safety tests. This would flag problem compounds before they enter pre-clinical testing in rodents.

4.1. Phenotype and GSH

The paracetamol concentration range 0–5 mM is consistent with the dose used to investigate paracetamol-induced liver injury in zebrafish in the literature. Furthermore, the gastrointestinal oedema phenotype in the *Xenopus* embryos was also observed in response to paracetamol in zebrafish larvae (Verstraelen et al., 2016). Similar to the *Xenopus* embryos, zebrafish larvae were exposed to soluble paracetamol in the media the embryos were swimming in. Furthermore, the zebrafish used

to model paracetamol-induced hepatotoxicity were aged of 72–120 hpf which is close to the *Xenopus* embryo age used in the research presented here (Verstraelen et al., 2016; He et al., 2013; Vliegenthart et al., 2014a; North et al., 2010).

For paracetamol-induced liver injury in humans, the reduced amount of free GSH is part of the pathophysiology mechanism as it allows the accumulation of the reactive metabolite NAPQI. The reduction of free GSH observed in the *Xenopus* embryos (Fig. 2) implies they have generated a small molecule from paracetamol, which depletes the free GSH store. The absence of the reduction in the embryos treated for only 24 h (Fig. 2B) indicates the GSH depletion could be time-dependent. Or it could be that at stage 38, for the first 24 h of the screen, the embryos do not have functional enzymes that are responsible for the production of the metabolites. The absence of metabolite generation could explain why the amount of free GSH has not been reduced. Future work should investigate the metabolic enzyme activity involved with *Xenopus* paracetamol production. Our preliminary work suggests that the embryos do generate similar paracetamol metabolites to humans (*i.e.* APAP-sulphate and APAP-glucuronide) (data not shown).

4.2. miR-122

Xenopus miR-122 has the same nucleotide sequence as zebrafish, humans and rodents and the WISH results show that miR-122 is liver-specific in the *Xenopus*, much like these animal models as well (Ahmed et al., 2015; Wienholds et al., 2005; Vliegenthart et al., 2014b). The presence of miR-122 in the blood in humans is an indication of DILI. The *Xenopus* embryos have a circulatory system at the ages used in this work, but the size of the embryos is too small to be able to easily obtain a blood sample, and the volume of blood within the embryo is very little. Consequently, in order to measure the expression of miR-122 with paracetamol treatment, it was decided to use the tail tissue to represent the blood or circulatory system. The tail contains a good vasculature and the liver tissue is not present in this body compartment. Under healthy circumstances there should not be a significant amount of miR-122 expression found in the tail. The size of the embryos means dissecting the liver alone would be difficult and time consuming. Therefore it was decided to dissect the whole gastrointestinal or gut region, including the liver, to represent the liver miR-122 expression.

miR-122 expression was normalised against miR-103 expression in the tissue of the embryos. Unlike U6, which is a small nuclear RNA (snRNA) that is traditionally used for miRNA quantification in qPCR, miR-103 is not affected by paracetamol treatment (Wang et al., 2013). The increase of miR-122 expression in the tail with increasing paracetamol concentration in Fig. 4 implies paracetamol-induced liver injury. This is comparable to adult zebrafish, which when exposed to a hepatotoxic dose of paracetamol, have a significantly high serum miR-122 expression (Vliegenthart et al., 2014b).The slight reduction of miR-122 expression in RNA isolated from gut tissue is potentially indicative of liver damage as fragmented WISH miR-122 staining in the liver indicates hepatocyte injury. However, there is no linear trend of miR-122 expression in the gut with regards to paracetamol concentration. It is hypothesised, this may be because miR-122 expression in the liver is so abundant, that a slight depletion cannot be detected. For future work, careful histological analysis of the liver to measure the presence of paracetamol-induced liver necrosis would need to be carried out to confirm this.

4.3. NAC

NAC is used to treat patients with a paracetamol overdose. Overall 3 incubation conditions were carried out with 0.5 mM NAC and 0–5 mM paracetamol: (Dart et al., 2006) 2 h NAC incubation prior to paracetamol addition, (Jaeschke, 2015) concurrent NAC and paracetamol treatment, and (Larson et al., 2005) NAC incubation in paracetamol-treated embryos for 24 h prior to harvest. Of the 3 conditions performed, the 24 h incubation prior to harvest is the most clinically relevant to humans. Embryos exposed to 1 mM paracetamol and then exposed to 0.5 mM NAC 24 h prior to harvest, had a significantly higher amount of GSH compared to embryos that were only exposed to 1 mM paracetamol (mean ± SEM; 97.1 ± 9.6 nmol/mg and 54.5 ± 6.6 nmol/mg respectively). However in all the treatment conditions, the amount of free GSH measured is greater in the embryos of the NAC treatment group compared with the embryos without NAC, of the same paracetamol concentration group. This smaller decline of free GSH with NAC treatment suggests the embryos are metabolising paracetamol to generate a small molecule that depletes GSH, and that this pathway can be reversed through the administration of NAC. This rescue mechanism implies (but cannot confirm) that the reactive metabolite generated in the *Xenopus* embryos is NAPQI, the same reactive metabolite produced in humans and other animal models.

The results are similar to the NAC response observed in zebrafish paracetamol overdose. In adult zebrafish treated with paracetamol, the amount of free GSH increased with NAC administration compared to zebrafish treated with paracetamol alone (North et al., 2010). Overall, these NAC response results indicate that *Xenopus* embryos are a promising model as they exhibit reactivity to human treatments.

4.4. AMAP: the non-toxic regioisomer of APAP

AMAP is a controversial, supposedly less toxic positional isomer to paracetamol, which has similar therapeutic indications to paracetamol, such as analgesic properties. In *Xenopus* embryos used here, the lack of significant difference between AMAP and paracetamol for the depletion of GSH indicates *Xenopus* could have a similar toxic reaction to AMAP as paracetamol. The embryos also have a similar concentration-dependent survival percentage for AMAP and paracetamol. This result does not necessarily diminish the integrity of the *Xenopus* as a toxicity prediction model, because toxicity associated with AMAP is not consistent across the existing animal models in drug development (Hadi et al., 2013).

5. Conclusion

Overall the *Xenopus* embryo model appears to have the ability to react to paracetamol in a dose-dependent manner as shown by looking at GSH levels and also by observing the expression of the liver-specific biomarker miR-122 in the embryo. This is comparable to research undertaken using zebrafish (Verstraelen et al., 2016; He et al., 2013; Vliegenthart et al., 2014a; North et al., 2010). The depletion of GSH with paracetamol treatment suggests the *Xenopus* embryo metabolic pathway is similar to that observed in humans and rodent paracetamol-induced liver injury models. To our knowledge, GSH reduction has not been measured in the zebrafish paracetamol-induced hepatotoxicity model. Going forward it would be interesting to confirm the mechanism of toxicity using a CYP450 inhibitor that is valid for *Xenopus*. Furthermore, the CYP450 isoform that could be involved in the *Xenopus* metabolism of paracetamol should be determined. Although the dominant isoform in humans that generates the reactive metabolite NAPQI is the CYP2E1, in zebrafish the CYP3 A65 isoform is predominantly responsible for NAPQI generation (Chng et al., 2012). At stage 38 to stage 45, the *Xenopus* embryos express CYP2D6 and CYP3 A4 as well as CYP2E1. In humans CYP2D6 and CYP3 A4 are responsible for over 50% of all drugs (Bertz and Granneman, 1997). In summary, these results represent the initial steps towards determining the advantages and limitations of the *Xenopus* embryo model as a predictive tool for DILI, and pave the way for future assessment of the value of *Xenopus* as an efficient and effective *in vivo* toxicity model for other organ systems.

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Competing interests

The authors declare they have no competing interests.

Transparency document

The Transparency document associated with this article can be found in the online version.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.toxlet.2018.09.016.

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