

# Differential Effects of CBZ-Induced Catalysis and Cytochrome Gene Expression in Three Dimensional Zebrafish Liver Cell Culture

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## Abstract

Two dimensional (2D) cell cultures have been the standard *in vitro* model for environmental safety research. However, it does not properly mimic *in vivo* system which hampered cellular proliferation and differentiation over an extended culture time and quickly lost its function. In this study, we constructed a new three dimensional (3D) zebrafish liver cell model that more closely mimics the *in vivo* micro-environment for pharmaceuticals metabolism studies. The magnetic levitation method was used to maintain the controlled and uniform 3D spheroids. These spheroids were then tested with carbamazepine (CBZ) treatment to determine the applicability of this assay in metabolism study. The results demonstrated that the 3D cell model is more resistant to CBZ over 2D cell culture and *in vivo* and 3D culture cells are noticeable in CYP1a1 activity and the concentration of carbamazepine-10,11-epoxide (CBZ-EP) than 2D cell culture. This 3D cell culture model will provide useful information toward the development of improved biomimetic organ functions for alternatives to animal testing in environmental contaminants.

**Keywords:** Zebrafish liver cell (ZFL); Three dimensional (3D) cells; Carbamazepine (CBZ) carbamazepine-10,11-epoxide (CBZ-EP); Cytochrome P450 1a1 (CYP1a1); Alternatives to animal testing

## Introduction

Concerns about the adverse effects of environmental contaminants have triggered research on zebrafish (*Danio rerio*), a vertebrate and one of the OECD toxicity testing organism, as a toxicity test model. The zebrafish is a promising species for evaluating chemical toxicity due to their rapid development, conserved molecular pathways and potential of high throughput screening [1-5]. The use of zebrafish also supports the 3Rs (reduction, refinement, and replacement) of animal use [6]. For these reasons, zebrafish liver (ZFL) cell lines can be used as a major target organ and adequately provided the unlimited supply of hepatocytes for metabolism testing at ease. In two dimensional (2D) cell cultures, ZFL has been shown the stable cytochrome P450 activities which can make the practical determination of chemical metabolism and toxicity. Among the CYP P450 subfamilies, CYP1a1 is the most well-known and sensitive to hepatocyte, which is a phase I drug metabolizing enzyme and a useful biomarker for analyzing the hepatocellular toxicity [7-9]. Therefore, the current use of the ZFL cell line as an *in vitro* model has proven to be efficient. However, 2D cell cultures tend to be extended in cell morphology, deplete hepatic polarity and lapse liver specific functions [7,10,11]. In addition, chronic chemical exposure could not be feasible in 2D cell culture due to the limitation of cellular surface and cell growths in long term. Cell growth in 3D spheroids generates endogenous extracellular matrix (ECM) surrounding the spheroids, while 2D monolayer has a homogenous cell layers probably with a contact with the culture plate. It has been shown that liver cell cultures in 3D models such as a hang in drop and a spinner bioreactor are significantly ameliorated the stability of CYP 450 expressions [12].

Carbamazepine (5H-dibenzo[b,f]azepine-5-carboxamide, CBZ) is an anti-epileptic drug and used for the treatment of epilepsy. With respect to the environmental issues, CBZ is of concern as one of the most frequently detected pharmaceutical residues in water bodies because this cannot be completely removed by the currently available water treatment systems [13-16]. Previous studies showed that environmentally relevant concentration of CBZ is not fatal to aquatic

organisms, whereas chronic effects of CBZ include sub lethal impacts to aquatic organisms, decreased fecundity of *Ceriodaphnia dubia*, and reduced population growth rates of *Daphnia pulex* [17-21]. However, studies on ecotoxicological data through toxicological assessment of CBZ are limited [22]. In general, *in vitro* method is recognized as one of the useful tools for the hazard and risk assessment of various chemicals in a fairly short time of exposure [23]. In this study, therefore, we developed 3D spheroids of ZFL cells with *in vitro* 3D hepatocyte model as a simple and robust format of toxicity testing for CBZ.

The major primary metabolite of CBZ, carbamazepine-10,11-epoxide (CBZ-EP), is an pharmacologically active metabolite of CBZ and an oxidized product formed by CYP450 enzymatic activity [24-27]. Regarding the metabolism of CBZ exposed to *in vitro* systems, we also aim for the monitoring of CBZ-EP as the primary metabolite. In addition, owing to the distinct differences on cell cultures between 2D and 3D of pharmaceuticals responses, we constructed 3D ZFL cell cultures using Nano shuttle methods. The different cell viabilities, CYP 450 inducibility and CBZ-EP metabolism were evaluated in this study. The acute hepatotoxicity responses to CBZ in zebrafish embryos were also studied for the potential allowances or limitations of these cell systems in environmental safety studies.

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## Materials and Methods

### Chemicals and reagents

Carbamazepine (CBZ) was purchased from LGC standards (Augsburg, Germany) and 10,11-dihydro-10, 11-epoxycarbamazepine (CBZ-EP), 10,11-dihydro-trans-10,11-dihydroxy-carbamazepine (DiOH-CBZ) and carbamazepine-d10 (CBZ-d10), were supplied from Sigma Aldrich (Steinheim, Germany). Acetonitrile and methanol, LC-MS grade, were purchased from VWR (Leuven, Belgium). Formic acid, ammonium acetate and dimethyl sulfoxide (DMSO) were provided from Sigma Aldrich. Stock solutions of CBZ were prepared in DMSO (10 mg/ml) and methanol (0.5 mg/ml) for the preparation of spiking solution to cell cultures and the analysis by liquid chromatography coupled with mass spectrometry (LC-MS), respectively. CBZ-EP and DiOH-CBZ stock solutions were prepared in methanol at a concentration of 10 mg/ml. All stock solutions were stored at -20°C before the preparation of working solutions which were diluted by a relevant solvent. Working solutions containing 60 mg/L of CBZ were made by appropriate dilution in medium just before spiking in 96-well plate resulting in a final concentration of 30 mg/L in each well with the total volume of the cell culture mediums of 200 µl for each well.

### Zebrafish embryo and ZFL cell cultures

Embryos at 8 hours post fertilization from a wild-type adult zebrafish (AB 1880-0-3) were maintained under a long photoperiod (16L: 8D light/dark cycle) at 26 ± 1°C water temperature in a climate room. The filtered tap water with Millipore 0.22 µm GSWP filter (Merck, Darmstadt, Germany) after sterilization at 120°C for 2 hours were used as a supplemented media. Embryo toxicity tests were conducted in 6 well cell culture plate (Greiner Bio-one, Frickenhausen, Germany) filled with 10 mL per each test solution, and were performed with 30 µg/ml of CBZ exposure test (3 replicates and 10 embryos per replicate). The mortality and the abnormality were recorded to assess the embryo toxicity based on phenotypic analysis. ZFL cells were purchased from ATCC (Wesel, Germany), which were maintained in Dulbecco's Modified Eagle Medium (DMEM) plus 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (P/S). After incubation, the cells were washed with PBS, trypsinized, and used for the spheroid formation. The ZFL cell line was maintained at 28°C in modified limit dilution factor (LDF) medium. ZFL were cultured in T-75 flasks. The phenol red-free LDF medium consists of 50% Leibowitz-15 and 50% DMEM/F12, supplemented with 7.5 mM Hepes (Sigma Aldrich, Steinheim, Germany) and 7.14 mM NaHCO<sub>3</sub> (Sigma Aldrich, Steinheim, Germany) and 1% penicillin-streptomycin-amphotericin (10,000 U/ml potassium penicillin, 10,000 µg/mL streptomycin and 25 µg/mL amphotericin B; Sigma Aldrich, Steinheim, Germany). When used for cultivation, 5% FBS, 10 µg/mL human recombinant insulin, and 5 µg/L mouse epidermal growth factor (Sigma Aldrich, Steinheim, Germany) were added. One day prior to exposure, the cells were treated with 0.25% trypsin-ethylenediaminetetraacetic acid (EDTA, Sigma Aldrich, Steinheim, Germany), suspended in complete medium, and spun at 300 × g in 5 min. The pelleted cells were resuspended in 67% supplemented LDF medium and counted using Trypan Blue (Sigma Aldrich, Steinheim, Germany) exclusion method, before seeded in 6-well cell plates (Thermo Scientific, Waltham, MA, USA) with 5 × 10<sup>5</sup> cells per well in 2 mL supplemented 67% LDF medium. In contrast to the cultivation medium, the 67% LDF exposure medium contained 6.7 µg/mL insulin and 10% charcoal-stripped and heat-inactivated FBS.

### 3D cell culture by magnetic levitation methods

The magnetic levitation (Nano3D bioscience, Huston, USA) was used to agglomerate cells into 3D cell cultures described by previous

report [28]. For spheroid formation, a density of 1 × 10<sup>5</sup> ZFL cells per 200 µL was obtained. When the growth of cell is more than 80%, the cell culture was treated in magnetic nanoparticles (n3D Biosciences, Houston, USA), for overnight. After detached, the cell were transferred in 24- or 96-well microplate at a density of 2 × 10<sup>6</sup> or 6 × 10<sup>4</sup> cells each well, which were magnetically levitated and cultured for four days. The culture medium was changed every two day. To examine the spheroid growth over time, 3D culture were captured from one to six days. The diameter of spheroid was measured and analyzed by image J software (SPSS, Inc, Chicago, IL, USA).

### MTT assay and live/cell staining

MTT assay (Invitrogen Inc., Waltham, MA, USA) was performed to examine ZFL cell cytotoxicity and proliferation with and without CBZ treatment. The ZFL cells were first seeded on 96-well plates with a cell density of 1 × 10<sup>4</sup> per well and incubated overnight to achieve around 80% confluence. The medium was then removed and the cells were exposed to serum-free medium with 30 µg/ml of CBZ from 0 h to 72 hours. After exposure, the serum-free medium with the test chemicals was removed and 100 µL medium with 5 mg/mL MTT was added to the wells and incubated for 4 hours. Supernatant was removed and 100 µL of DMSO was supplemented to each well incubated for 10 min. Absorbance of samples was measured using a microplate reader (TECAN, Zurich, Switzerland) at 570 nm. Cell viability of the ZFL spheroids was examined by a Live/Dead cell assay kit (Invitrogen Inc., Waltham, MA, USA). 1 µM calcein-AM and 1 µM ethidium homodimer were supplemented to the cell culture dish and incubated for 10 min at 37°C. After washing with PBS, the cells were visualized with fluorescent microscopy (Olympusmicro, Shinjuku, Japan). To examine the initial cell distribution within the spheroids, cells were labeled with 1 µM 5-chloromethyl fluorescein diacetate (CMFDA, green cell tracker dyes, Invitrogen Inc., Waltham, MA, USA) for 15 min prior to the spheroid formation.

### cDNA synthesis and real time polymerase chain reaction

All samples from ZFL cells and embryos exposed to 30 µg/ml of CBZ as described above were collected for total RNA extraction using the Trizol reagent (Sigma Aldrich, Steinheim, Germany). For each samples, 0.3 ml of supernatant of ZFL cell in culture media was mixed with 0.7 ml of Trizol reagent and 0.2 ml of chloroform. After centrifugation at 12,000 g for 10 minutes, the precipitated RNA in the aqueous solution was collected and washed with 70% ethanol and finally dissolved in 20 µl of DEPC-treated water. To synthesis DNA templates for CYP and β-actin, the primers were designed and the cDNAs were amplified in real-time PCR by the primer pairs using SYBR Green PCR kit (Invitrogen Inc., Waltham, MA, USA). The reaction was carried out with one cycle of reverse transcription at 45°C for one hour, followed by 40 cycle of amplification (15 sec at 95°C, 30 sec at 60°C and 30 sec at 72°C) and a final extension of 5 min at 72°C in a real time PCR system (Applied Biosystems, Foster City, CA, USA). The PCR sequences are followed as Table 1.

The relative gene expression of each samples were calculated as described using the formula of fluorescence intensities (2<sup>-ΔΔC<sub>t</sub></sup>) in

Name	Sequence (5' -> 3')
<i>cyp 1a1_F</i>	CGCTTGATGGGCTTGCTCT
<i>cyp 1a1_R</i>	CGCAGCTAAACAGGCACTC
<i>β-actin_F</i>	TGTCCTGTATGCCCTTGTT
<i>β-actin_R</i>	AAGTCCAGACGGAGGATGG

**Table 1:** Nucleotides sequences of primers in CYP 1a1 and β-actin expression.

SPSS statistics computing software. Data are expressed as means  $\pm$  standard error of means (SEM) (n=3 per exposure concentration). Electrophoresis of each PCR product in 1.0% LE agarose gel was performed and following SYBR green staining, the results were viewed under the GelDoc image-analysis system (Bio-Rad, Hercules, CA, USA). Quantitative real-time PCR was performed on an iQ5 Multicolor Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA). Results were further normalized using the calibrator cDNA of the positive control.

### Extraction of target chemicals

A model chemical, CBZ, and its major metabolite, CBZ-EP, within the tested systems were extracted before analysis. The samples were collected at 0, 24, 48, 72 hour of exposure to analyze target chemicals. A 50  $\mu$ l aliquot of stored sample was mixed with 230  $\mu$ l of methanol. The mixture was then vigorously vortexed for 10 seconds and centrifuged at 13,000 g for 20 minutes. 250  $\mu$ l of methanol was then added to each sample and spun down again at 13,000 g for 20 minutes. After centrifugation, 100  $\mu$ l of the supernatant was transferred to HPLC vial and diluted 10 times with methanol. 20  $\mu$ l of CBZ-d10 was spiked in each vial as internal standard (nominal concentration of 50 ng/ml) and stored at -20°C until analysis.

### Liquid chromatography – mass spectrometry

CBZ and CBZ-EP were analyzed by LC-MS/MS with Jetstream electrospray ionization (ESI). Liquid chromatography was performed by an Agilent 1290 series LC system (Agilent Technologies, CA, USA). Separation of the compounds was performed on a Zorbax Rapid Resolution High Definition column (2.1 mm  $\times$  50 mm, 1.8  $\mu$ m particle size, Agilent, Stuttgart, Germany) maintained at 30°C. To protect the chromatographic column, a C18 guard column (Zorbax Eclipse Plus 2.1 mm  $\times$  5 mm, 1.8  $\mu$ m, Agilent, Stuttgart, Germany) was equipped with the column. A binary gradient consisting of two mobile phases, Milli-Q water containing 5 mM ammonium acetate and 0.1% formic acid (solvent A) and LC/MS grade acetonitrile (solvent B), was used. The initial conditions were 95% A and 5% B, and B was increased to 20% in 4 minutes followed by increasing to 100% over 3.45 minutes. This condition was then held for 3.55 more minutes. The equilibration time was following for 1 more minute, which resulted in a total run time of 13 minutes including a post-run protocol. The flowrate was kept at 0.4 ml/min and the sample injection volume was set at 5  $\mu$ l. Mass spectrometry was performed using an Agilent 6460 triple-quadrupole mass spectrometer which was operated with ESI in positive mode. Mass spectrometric conditions include: gas temperature of 350°C, gas flow of 10 L/min, nebulizer gas of 35 psi, capillary voltage of 4000 V and source temperature set to 400°C. Nitrogen gas supplied by the nitrogen generator (F-DGSI, Evry, France) was used as a drying and a nebulizing gas. The MS analysis in multiple reaction monitoring

(MRM) mode was carried out using instrument setting described in Table 2. The accurate mass of precursor ions and the transitions to two product ions were monitored. The most abundant transition is used for quantification, whereas the second one is used to confirm the target analyte. Product ions with higher intensity were m/z 194.0 for CBZ and m/z 179.8 for CBZ-EP, which is in accordance with the previous study determined CBZ in human plasma using LC-MS/MS [29]. These source and fragmentation parameters were optimized for each analyte using 100 pg/ml of a single standard dissolved in methanol, by passing the same column explained in the method. Calibration curves were established at nine levels for each analyte ranged from 1.95 to 500 ng/ml with a correlation coefficient  $R^2 > 0.99$ . The concentrations of all measurements were recalculated according to the appropriate dilution factors. Instrumental limit of detection (ILOD) and instrumental limit of quantification (ILOQ) values were estimated from the low concentration calibration standard giving a signal-to-noise ratio of 3:1 and 10:1, respectively. ILOD values of 120 pg/ml for CBZ and 129 pg/ml for CBZ-EP were achieved. Agilent MassHunter Qualitative Analysis and Quantitative Analysis software (v.B.06.00) were used for data processing.

### Extraction yield

Preliminary experiment to evaluate the extraction recoveries was performed with supplemented medium at concentrations of 10 and 2  $\mu$ g/ml for CBZ and CBZ-EP, respectively (n=3). Previously described extraction procedure was applied to prepared samples and injected into the LC-MS/MS system for the analysis. Extraction recoveries of target compounds from this method are in Table 3.

### Statistical analysis

All data are expressed as mean  $\pm$  standard error of mean (SEM). A two-way analysis of variance (ANOVA) was used to assess the differences in *in vitro* and *in vivo* system for CBZ-EP determination. Statistical significance was set at  $p < 0.05$ .

## Results and Discussion

### Morphology of 2D and 3D cell culture

The morphologies of 2D cell and 3D cells were observed with bright field microscopy as shown in Figure 1. Cell morphology was investigated from day 2 to day 6 after cell seeding. Bright field microscopy showed that 2D cells were spread and forming monolayer with irregular cell morphology, whereas the cells in 3D were with spheroid formation. Uniform 3D spheroids with the diameter between 350 and 400  $\mu$ m were obtained after two days culture by magnetic levitation methods. The 3D cell culture became to form spheroid in suspension and initially decreased diameter and transparency due to compact cellular reorganization. The spheroid diameter then increased continuously after two days culture.

Compounds	RT (min)	Transitions	FV (V)	CE (V)	ILOD (ng/L)	ILOQ (ng/L)
CBZ	7.2	237.0 $\rightarrow$ 194.0 (quantifier)	80	20	120	399
		237.0 $\rightarrow$ 165.0 (qualifier)	80	50		
CBZ-EP	6.6	253.1 $\rightarrow$ 179.8 (quantifier)	85	36	129	429
		253.1 $\rightarrow$ 235.8 (qualifier)	85	8		
CBZ-d10 (Internal standard)	7.1	247.2 $\rightarrow$ 203.9 (quantifier)	110	40	N.A.	N.A.
		247.2 $\rightarrow$ 201.9 (qualifier)	110	20		

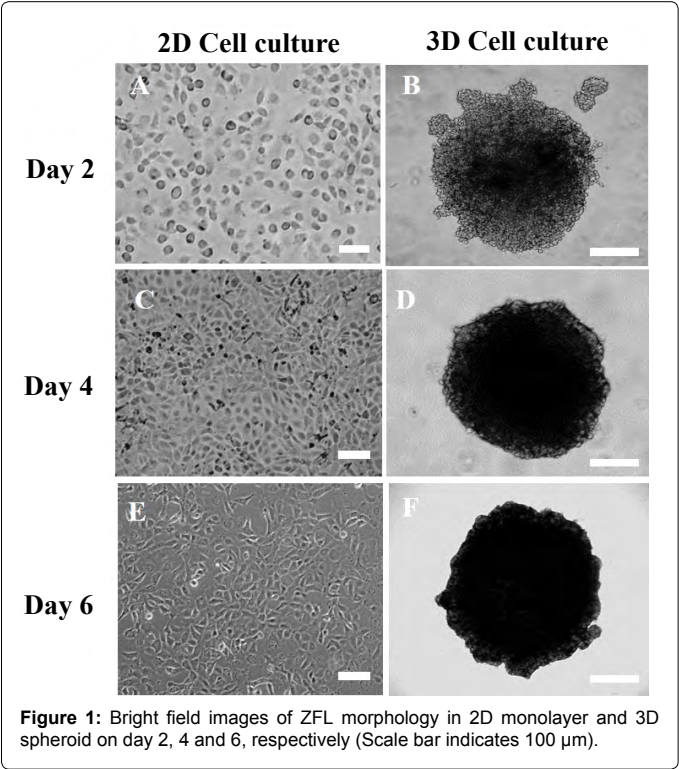
N.A.: Not Applicable

**Table 2:** LC-MS/MS conditions applied in a positive mode for the quantification of compounds. Retention Time (RT), MRM transitions selected, Fragment Voltage (FV), Collision Energy (CE), Instrumental Limits of Detection (ILOD) and Quantification (ILOQ) are indicated in this table.



	Sample	2D cells	3D cells	Zebrafish Embryo
CBZ-EP recovery (%)	<i>In vitro</i>	97.10 (± 3.93)		N.A
	<i>In vivo</i>	N.A	N.A	105.71 (± 4.29)
CBZ-EP concentration (ng/ml)	Day 0	N.A	N.A	0.15 (± 0.01)
	Day 1	0.12 (± 0.01)	0.10 (± 0.07)	2.45 (± 0.29)
	Day 2	0.16 (± 0.02)	0.21 (± 0.06)	3.99 (± 0.14)
	Day 3	0.22 (± 0.01)	0.35 (± 0.03)	5.04 (± 0.54)

**Table 3:** CBZ-EP concentration (ng/ml) in time course following a single dose of 30 µg/ml CBZ. All data enlisted are the average of triplicate and standard deviation from mean.



**Figure 1:** Bright field images of ZFL morphology in 2D monolayer and 3D spheroid on day 2, 4 and 6, respectively (Scale bar indicates 100 µm).

Expression of liver specific Cytochrome P450 gene

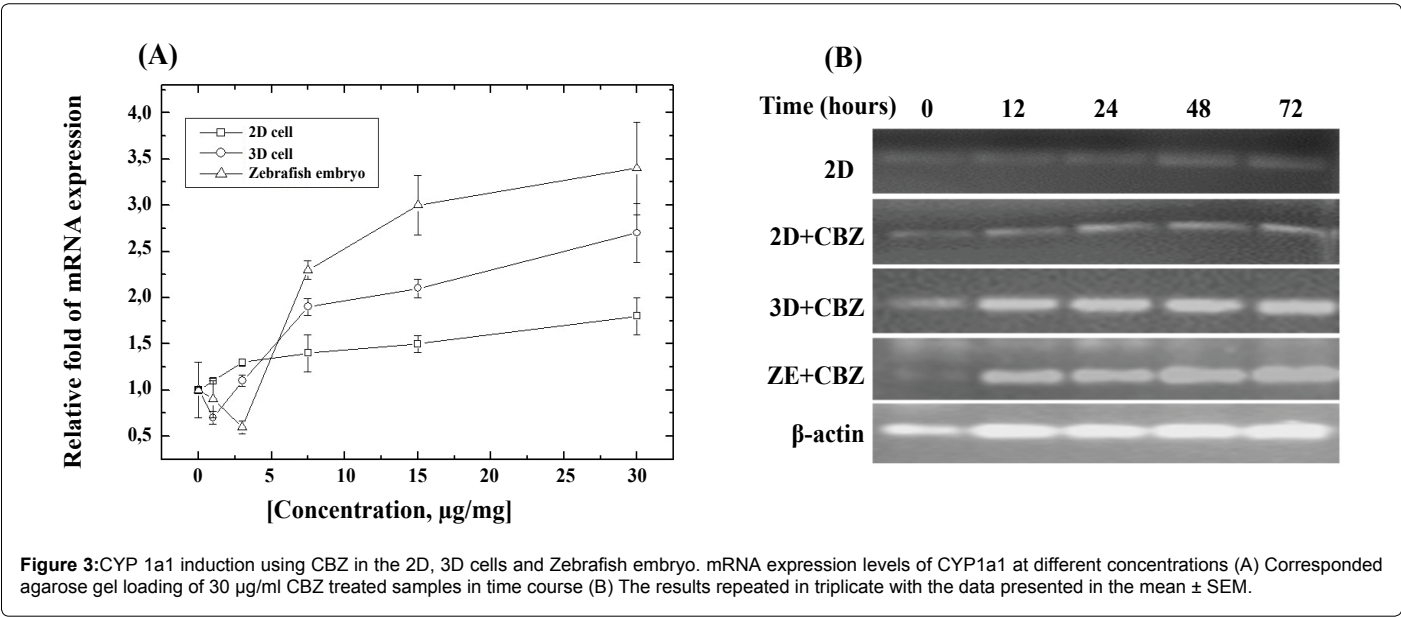
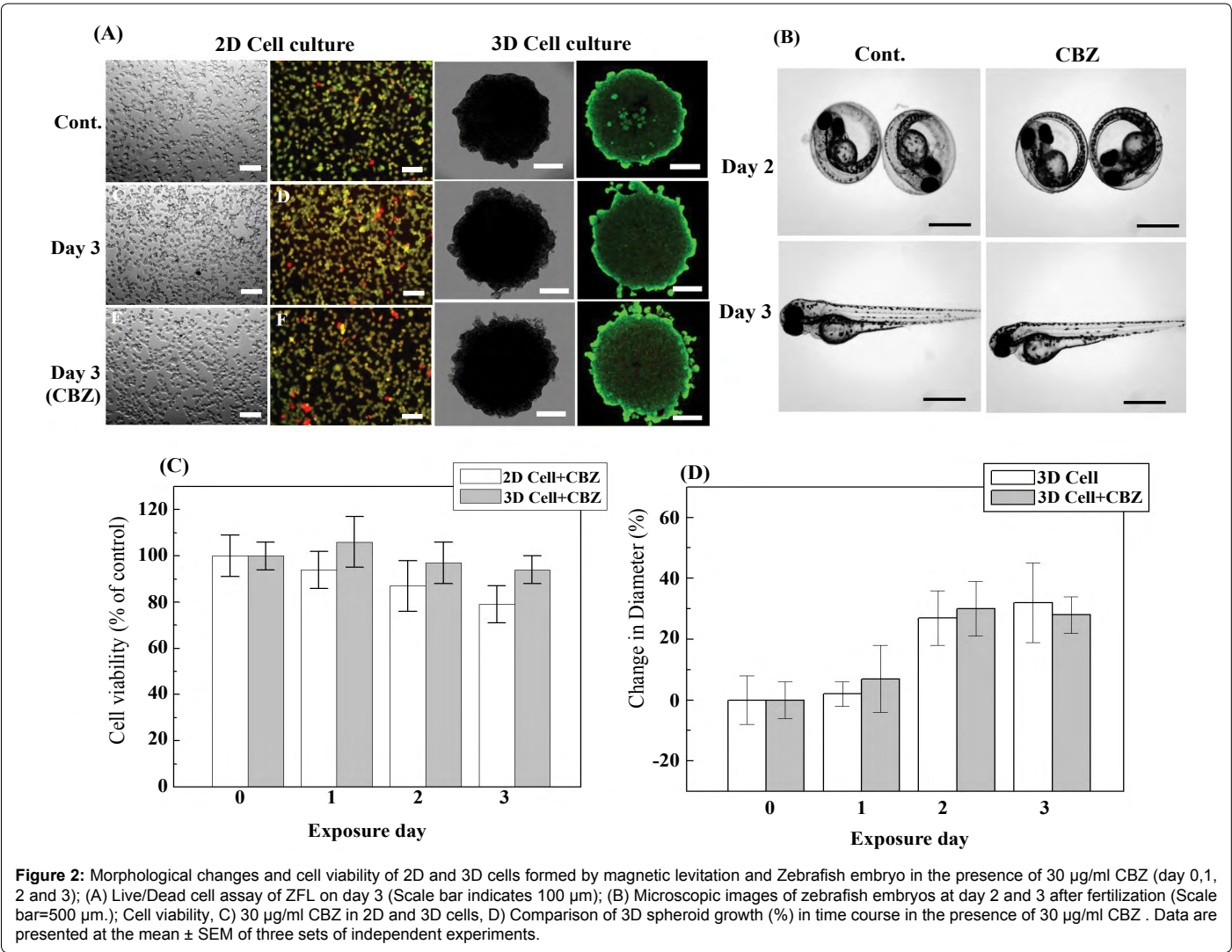
CYP 450 induction is one of the most important catalytic metabolism for pharmaceutical function and xenobiotics [30]. The mRNA levels of CYP1a1 were measured by RT-PCR at different concentration for 3 days. As shown in Figure 2A), CYP 450 levels of 2D cells, 3D cells and Zebrafish embryo were comparable and higher in the 3D cell and Zebrafish embryo than those in 2D monolayer from the point of 7 µg/ml CBZ. Fold changes of CYP1a1 mRNA reached saturation peak around 30 µg/ml CBZ, exhibited by 1.5 fold for 2D cell, 2.5 fold for 3D cell and 3.25 fold for Zebrafish embryo at day 3 of exposure compared to basal level of CYP1a1 at day 0, respectively. Hence, 30 µg/ml treatment was fixed to treat all experimental groups in this work. This concentration is also meaningful because it was found as no observed effect concentration (NOEC) for Zebrafish embryo by van den Brandhof et al. [31]. Subsequently we investigate of CYP1a1 mRNA levels on day 3 following a single dose of 30 µg/ml CBZ in 2D cell, 3D cell and Zebra embryo (Figure 2B). After 30 µg/ml CBZ exposure, we observed CYP1a1 levels of 3D and Zebrafish embryo dramatically increased those in 2D cell from the 12 hours, which is significant biomarker for *in vivo* liver. Quite interestingly, the 3D cell cultures exhibited a relatively higher mRNA expression at initial time whereas, undeveloped zebrafish embryo exhibited low levels of mRNA at 0 time that 3D cell could be regarded as a key biomarker of mature hepatocytes of adult Zebrafish.

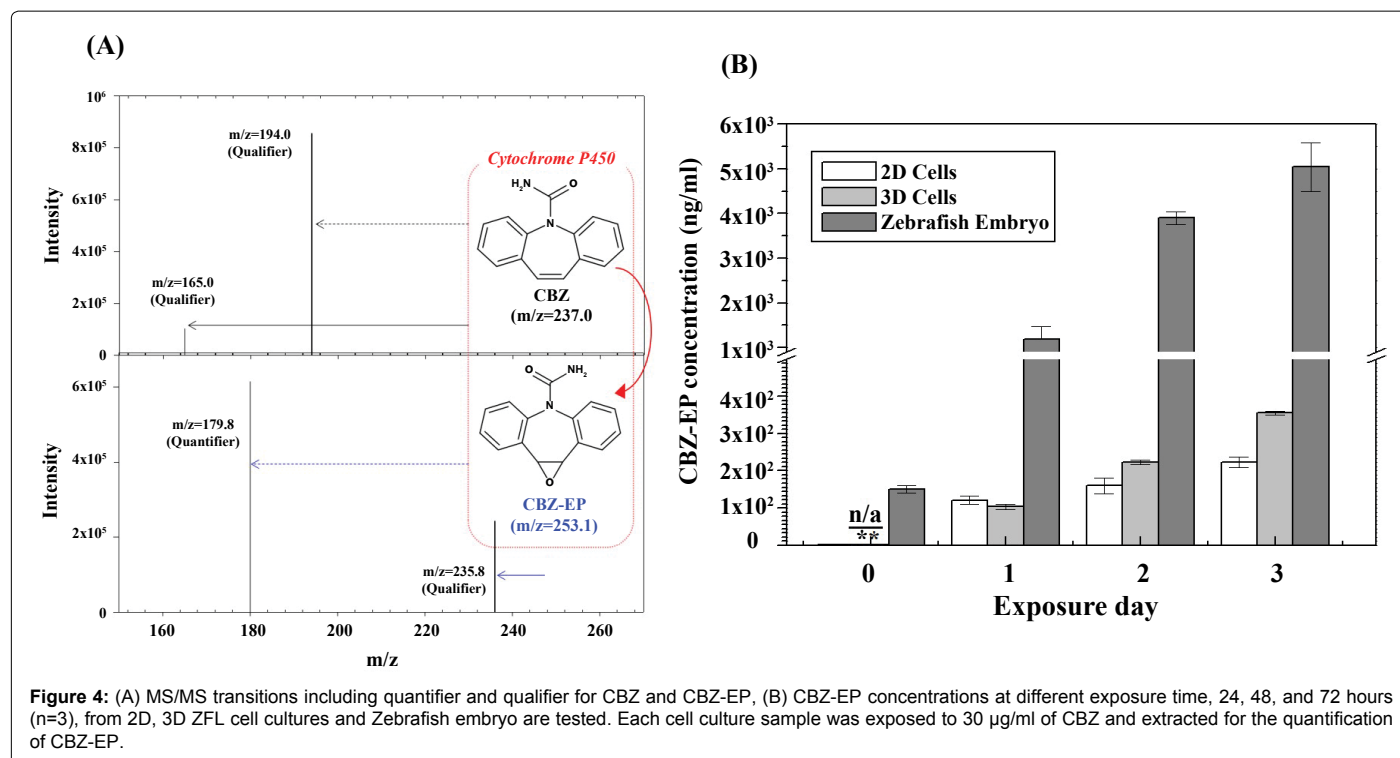
2D, 3D Cell and Embryo changes in morphology and viability

Cell viability was determined in both 2D and 3D cell cultures albeit morphological changes of embryo in the presence of 30 µg/ml CBZ. As shown in Figure 3A, live/dead cell staining was performed with 1 µM calcein-AM and ethidium homodimer to examine the cell viability. Highly compact 3D cell spheroid were formed with live cells even in the presence of 30 µg/ml CBZ. Most of cells and only few cells turned to red for dead cells, whereas higher 2D cell death were observed both in the presence and absence of 30 µg/ml CBZ exposure at day 3. There is no morphological change in both control and 30 µg/ml CBZ exposure group (Figure 3B), indicating that the exposure to 30 µg/ml CBZ has non-toxic effect on zebrafish embryo. MTT assay exhibited higher cell viability in 3D cell culture than those in 2D cell culture 30 µg/ml CBZ exposures as shown in Figure 3C. Over the three days of 2D cell cultures, treatment with 30 µg/ml CBZ resulted in 90%, 84% and 79% cell viability on day 1, 2 and 3, respectively. By contrast the 3D cell culture with 30 µg/ml CBZ exhibited no significant change in cell viability and resulted in 105%, 98% and 95% on day 1, 2 and 3, respectively. It demonstrated that 3D cell culture has higher CBZ resistance than 2D culture. In line with those results, spheroid diameter increased by 20% for CBZ treated 3D cell culture and 35% for control in day 3, indicating that there were no statistically significant differences over the 3 days of exposure with cell culture shown in Figure 3D. It can be concluded that 3D cell culture and healthy zebrafish embryo has similar CBZ resistance at 30 µg/ml. Higher CBZ resistance can be explained by CBZ diffusion through the extracellular matrix may be hampered in the 3D cell spheroid, which leads to decreased drug delivery to inner layer and lower toxicity than 2D cell and tight cell to cell interaction may improve cell viability and proliferation.

Transformation of CBZ to CBZ-EP

It is well known that the hepatocytes convert CBZ to CBZ-EP, one of the primary metabolites, and further to DiOH-CBZ [9,30,32,33]. DiOH-CBZ in all samples were not detected. Therefore, to validate catalytic activities of CBZ on 2D and 3D cell cultures as well as zebrafish embryos, quantification of CBZ-EP in samples at different exposed times of 0, 1, 2, and 3 days was performed by using LC-MS/MS and the results were shown in Figure 4 and Table 3. The chemical extraction procedure was successfully applied to all sample preparations. Applied procedure for all samples appeared to produce the fairly consistent and reproducible recoveries as shown in Table 3. The concentrations of CBZ-EP for tested 2D and 3D cell cultures at 0 Day were not applicable due to their lower values than the detection limit (Figure 4 and Table 3). Interestingly, Zebrafish embryo at 0 day resulted in 5.04 µg/L of CBZ-EP. It can be assumed that CYP P450 in lethal embryo at 0 day was functionalized and maintain their CBZ binding activities and rapid turnover epoxidation of CBZ during sample preparation at room temperature. For all samples, the amount of CBZ-EP was increased continuously from day 1 as the results of quantitative analysis (Figure 4B). Time dependent and rapid CBZ metabolism presented in this





**Figure 4:** (A) MS/MS transitions including quantifier and qualifier for CBZ and CBZ-EP. (B) CBZ-EP concentrations at different exposure time, 24, 48, and 72 hours (n=3), from 2D, 3D ZFL cell cultures and Zebrafish embryo are tested. Each cell culture sample was exposed to 30 µg/ml of CBZ and extracted for the quantification of CBZ-EP.

study has been reported previously [8]. CBZ treatment clearly induced higher epoxidation activities in zebrafish embryo (5.04 µg/L per 10 embryos) than 2D (0.22 µg/L per 1 × 10<sup>6</sup> seeding cells) and 3D (0.30 µg/L per 1 × 10<sup>6</sup> seeding cells) cell culture at last sampling point (the values of day 3 in Table 3). It is obvious that 3D cell culture had higher metabolic activities for CBZ as showing higher conversion values of CBZ-EP compared to 2D cell culture. Gagne et al. described that CBZ may induce rather CYP2 or CYP3 genes, not CYP1s under *in vitro* condition of piscine species [34]. However, the expression of CYP3a65 in this study did not show clear gene expression in time dependency in the presence of 30 µg/ml CBZ (SI). It can be assumed that mRNA level of CYP 450 (CYP3a65) is the hepatotoxicity biomarker but might not be deeply involved in epoxidation of CBZ. Accordingly, the epoxidation of CBZ can be mainly induced by the other subfamily of CYP 450 enzyme. The higher CBZ-EP levels in embryo provide the evidence to support notion that liver specific CBZ-EP metabolism were improved in the 3D cell cultures compared with 2D cell cultures.

## Conclusions

In this study, ZFL 2D, 3D cell culture and Zebrafish embryo were compared to evaluate the potential of 3D cell culture as an *in vitro* hepatic toxicity and metabolism study. Culturing ZFL cells into 3D spheroids can be promoted to overcome the limitation of 2D *in vitro* culture and provide similar CBZ metabolism with zebrafish embryo assay because 3D cell culture recapitulates the dimensionality of the liver tissue, hence it provided functions more similar to an *in vivo* model. The higher cell viabilities, particularly with respect to high inducibility of CYP 450 metabolism, is important for organotypic function for the continued cell proliferation and dimensionality when compared to Zebrafish embryo. The 3D cell culture developed in this study can be applicable to the environmental toxicity testing. Our results also provided the feasibility of 3D cell culture in high throughput screening toward the development of alternative to animal model for chemical safety screening.

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