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Original Article

ITE enhances the CYP450s activities of rat hepatocytes in a bioreactor

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Abstract: Actually all hepatocyte cell lines are lack of major cytochrome P450 (CYP)-related enzyme activities, making them unrepresentative of in vivo hepatocytes. To evaluate the effects of 2-(1'H-indole-3'-carbonyl)-thiazole-4-carboxylic acid methyl ester (ITE) on liver-specific functions of rat hepatocytes we conducted this study. In this study, rat hepatocytes were treated with 2-(1'H-indole-3'-carbonyl)-thiazole-4-carboxylic acid methyl ester (ITE), an endogenous nontoxic ligand for aryl hydrocarbon receptor, in monolayer cultures and as microspheres in a bioreactors. The viability and mRNA as well as metabolic activities of drug metabolizing enzymes were determined. Results: When rat hepatocytes were cultured on monolayer, in microspheres and a fluidized bioreactor, ITE enhanced the liver-specific functions, including urea synthesis as well as CYP1A2 and CYP3A4 activities. The transcription levels of several CYP450-related genes were also increased in cells treated with ITE in these three culture conditions. Furthermore ITE showed little toxicity as the viability of rat hepatocytes was not affected by ITE. ITE enhanced CYP1A2 and CYP3A4 enzymatic activity and transcription levels of metabolic related genes of rat hepatocytesin three culture conditions above, indicating that it may be utilized for future cell applications.

Keywords: CYP450s, ITE, metabolic functions, microspheres, rat hepatocytes

Introduction

The liver plays many essential roles in maintaining normal physiology. Therefore, in many occasions, such as bioartificial liver and fundamental cell biology studies, liver cells with high functions have been frequently used for various purposes [1-4]. In order to complete these applications, it is important to maintain cells with high expression and long-term of liver cell functions during culturing in vitro. It is acknowledged that liver-specific functions and proliferation of primary hepatocytes are rapidly lost during culturing [5], and that hepatoma or hepatocellular carcinoma (HCC)-derived cell lines also need to be dramatically manipulated to retain liver-specific functions and safety-related requirements [6]. Therefore, it has been a challenge to maintain viable and functional hepatocytes for extended periods of time [7, 8].

The functions of hepatocytes mainly contain biosynthesis and detoxification. Hepatic detoxification is a process of the biotransformation which converts hydrophobic toxins into water-soluble substances thus secreting into urine or bile. It encompasses 4 phases: hepatic uptake, intracellular modification, subsequent conjugation, and secretion. As a typical hydroxylation reaction, intracellular modification is conducted by the family of CYPs. The CYP3A family is engaged in metabolizing approximately 50% of all xenobiotics and considered to be the most dominant one at the level of both expression and potency [9].

To address the *in vitro* culturing challenges, we introduced a novel method by employing ITE, a tryptophan derivative that acts as an endogenous aryl hydrocarbon receptor (AhR) ligand [10], into the culturing medium. AhR is a transcription factor that increases xenobiotic metabolism, histone modification, and tumorigenesis [11]. Meanwhile, AhR also can regulate the expression of several isozymes of CYP450 drug-metabolizing enzymes [12]. Moreover, be-

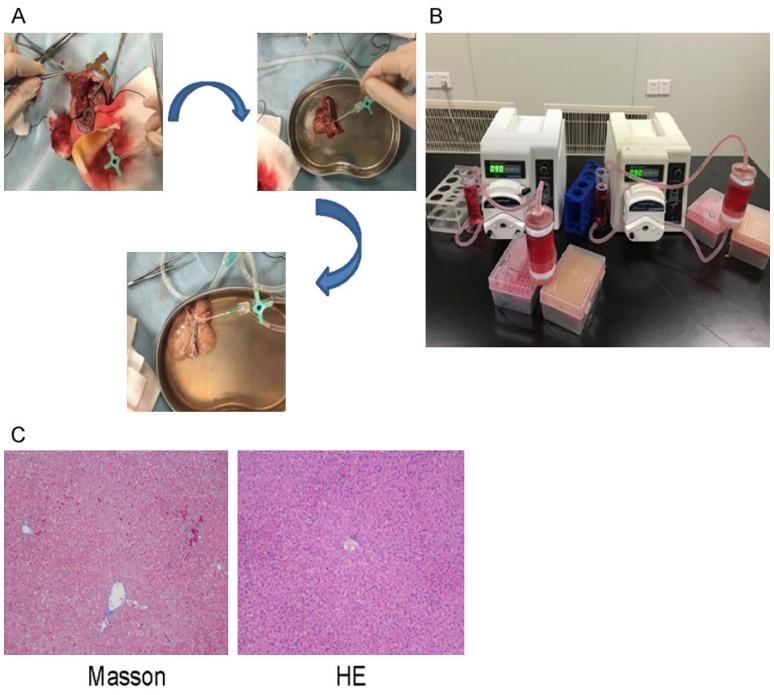

Figure 1. Rat hepatocytes co-cultured with ITE in fluidized bed bioreactor. A. The process of isolating rat hepatocytes from the whole liver of an adult SD rat. B. The chart showed cell-containing microspheres were fluidized at 90 mL/min to generate adequate perfusion conditions within the bioreactor. The whole circulation operated continuously for 48 h under this condition. C. Histological images of rat livers were stained with Masson and HE (magnification ×100).

ing isolated from porcine lung tissue [11], ITE has been identified as a very potent endogenous agonist for AhR. Compared with 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), another potent but man-made ligand of AhR [12], ITE has no discernible toxicity as reported in the literature so far [11].

Herein we cultured rat hepatocyte with ITE on monolayer, microspheres in static condition and in a fluidized bioreactor. We compared the functions of rat hepatocytes with/without ITE by characterizing its hepatic functionality, biosynthesis, proliferative capacity and transcription levels of genes in these three culture conditions above. In our previous study, investigations had focused on the effect of ITE on hepatocellular carcinoma cell candidates of BAL application and its related mechanism.

Materials and methods

Reagents and antibodies

ITE was a gift from Dr. Jiasheng Song, AhR Pharmaceuticals, Inc. Since ITE was dissolved in dimethylsulfoxide (DMSO), the control group 0 μM ITE represented that control media contained same quantity of DMSO. Therefore, 0 μM ITE as control group was chosen for the rest of the experiments described below.

Rat hepatocyte culture

Rat hepatocytes were isolated from the whole liver of an adult Sprague Dawley (SD) rat (male, 7-8 weeks old and weighing approximately 210 g) by performing liver perfusion using 0.84% dispase and 0.05% collagenase (Thermo Fisher Scientific 81 Wyman Street Waltham, MA USA) (**Figure 1A**). All studies on mice were carried out according to the National Institute Guide for the Care and Use of Laboratory Animal. The animal protocol has been approved by the Committee of the Ethics of Animal Experiments of the Zhejiang University.

The cell viability was measured by the trypan blue exclusion method, and cells with more than 85% viability were used for the subsequent experiments. The isolated hepatocytes were cultured in Dulbecco's Modified Eagle Medium (DMEM) (12430, GIBCO, Auckland, NZ) supplemented with 10% (v/v) fetal bovine serum (FBS) (10099, GIBCO, Grand Island, NY) as well as 1% penicillin/streptomycin (GIBCO, Auckland, NZ).

For the monolayer condition, hepatocytes (5×10^4/well) were inoculated onto a 24 well cell culture cluster (3524, Costar, Corning Incorporated, NY, USA) coated with type I collagen (Thermo Fisher Scientific 81 Wyman Street Waltham, MA USA). And the microsphere condition was described in detail in the Alginate Microsphere Production part. All the cells were cultured at 37°C in a humidified atmosphere of 5% CO_2 and 95% air.

Immunohistochemistry

For the immunohistochemistry study the left liver from the perfusion was immediately fixed

by immersion in 10% paraformaldehyde diluted in phosphate-saline buffer (PBS), dehydrated in graded ethylic alcohol, and embedded in paraffin. Sections 5 μm thick were stained with hematoxylin/eosin and Masson trichrome.

Cell viability test

Cell viability was determined using the MTT assay (Roche, Basel, Switzerland; 11465007-001). Briefly, cells were cultured with 100 μl of medium per well in 96-well microplates. After adding 10 μl of the MTT labeling reagent (final concentration 0.5 mg/ml) to each well, plates were incubated for 4 h in a humidified atmosphere at 37°C and 5% CO_2. Plates were incubated overnight with 100 μl of the solubilization solution in each well. Since formations of purple formazan crystals were proportional to the number of metabolically active viable cells, absorbance was measured using a microplate reader (Beckman Coulter, Brea, CA; DTX880) at a wavelength of 570 nm.

Cell-microcapsule production

According to the previously described [13] with modifications we conducted the alginate encapsulation with the isolated rat hepatocytes. Briefly, hepatocytes were suspended in 2% sodium alginate solution at a density of 3.0×10^6/ml (154 mM NaCl, 10 mM HEPES, pH 7.4). Each mixture was sprayed at 9.5 ml/min through a 300 micrometers nozzle using an electrostatic microencapsulator unit (Nisco Engineering, Zurich, Switzerland). The vibration frequency of the nozzle was kept at 0.30 kHz. The alginate droplets were collected in a calcium chloride gelation bath (154 mM NaCl, 10 mM HEPES, 115 mM $CaCl_2$, pH 7.4), followed by 10 min of gelling and normal saline washes for three times. Eventually, rat hepatocytes microspheres with the designed 800 micrometers diameter were produced.

Fluidized bed bioreactor and dynamic perfusion

The fluidized bed bioreactor was designed for application in our previous study [14]. In this study, a bioreactor with smaller size was produced. The parameters of the bioreactor were: bottom diameter: 40 mm, top diameter: 40 mm, and height: 55 mm (inner size of the container). Additionally, the membrane filters (300 mesh/inch) were attached to the bottom and the top to prevent microspheres from entering the circulation. Dynamic perfusion of encapsulated cells was performed inside a closed-loop circuit incorporating fluidized bed bioreactor. After cells were encapsulated in alginate microspheres, 20 ml rat hepatocytes microspheres with/without ITE were placed inside the bioreactor in fluidized culturing. The final volume of the circulating culture medium was 150 ml. Cell-containing microspheres were fluidized at 90 ml/min to generate adequate perfusion conditions for the greatest bed expansion within the bioreactor. The whole circulation operated continuously for 48 h under this condition, as shown in **Figure 1B**.

Assessment of cell viability by confocal microscopy of fluorescein diacetate (FDA)/propidium iodide (PI)-stained cells

Microspheres were washed twice with DMEM medium without phenol red and then stained for 2 min with 5 mg/ml FDA and 10 mg/ml PI in Opti-MEM medium without phenol red (Gibco/Invitrogen, Paisley, Scotland). The stained beads were washed twice with Opti-MEM medium and examined using a Biorad Radiance 2100 confocal microscope (Biorad, Hertfordshire, U.K.). Images were captured and analyzed using Lasersharp 2000 software (Biorad).

Metabolic activity assay of CYP450 enzymes

Various groups of cell culture models were used in our study that include: 2D, 3D, and 3DF. CYP450 1A2, 3A4 enzyme activity assays were carried out directly in 24-well plates. The measurement of luciferase activity was performed with a P450-Glo CYP1A2 assay (V8422; Promega, Madison, WI) and a CYP3A4 assay (V9002; Promega, Madison, WI). In short, cells were incubated at 37°C in Krebs-Henseleit buffer containing Luciferin-1A2 and in fresh medium containing Luciferin-IPA, respectively. After 1 h incubation, 50 μl of buffer or culture medium from each well were passed to a 96-well opaque white plate by mixing with an equal volume of the luciferin detection reagent to initiate a luminescent reaction. After 20 min shaking at room temperature, luminescence was measured using a microplate reader (DTX880; Beckman Coulter, Brea, CA).

Table 1. Primers used in the real-time quantitative PCR analyses

Name	Forward	Reverse
E-cadherin	5-GCTGGAGACCAGTTTTCTCG-3	5-TCTGGCCTGTTGTCATTCTG-3
Connexin32	5-CCTCAAGCCGTAGCATTTTC-3	5-AATGAGGCAGGATGAACTGG-3
Albumin	5-ATACACCCAGAAAGCACCTC-3	5-CACGAATTGTGCGAATGTCAC-3
CYP1A2	5-TGCTCAACCTCGTGAAGAGCAGC-3	5-TGGCAGCTGGGGTCTGTCAGAA-3
CYP2E1	5-CATGGAAGGATGTGCGGAGGTTTTC-3	5-GCAGGGTGCGCAGCCAATCA-3
CYP3A2	5-AGCTCTCACACTGGAAACCTGGGT-3	5-GGGCCAAAATCCCGCCGGTT-3
HNMT	5-TGGATTCCGCCTACCCAGGGA-3	5-TCCTCTTTGAGGTCCAGTGGTGCTG-3
NNMT	5-GGTCCAGGCACTGTGCAGAAAATGAG-3	5-GAGGGCAGTGCGATAGGCCG-3
TPMT	5-GGGTGACCCGTCACATCGCAT-3	5-TGGCGGGCCTGTGTGTTTTGT-3
GST	5-ATAGAAGCCCTGCTGTCCAA-3	5-GTGGTGAACGCCACTTTCTT-3
NTCP	5-TCCAAGCTGCAGACGCACCATC-3	5-TGGGTACCTTTTTCCAGAGCTGCTG-3
OCT1	5-ACCTGTTCCGCACTCCCAACCT-3	5-AGGGCAGAGCATACCATCATCCCA-3
OATP1A4	5-AGGCCCTGACTGTGCCAACAA-3	5-ACTCAATGTCCCCAGGGAACTGG-3
GAPDH	5-ATCACTGCCACTCAGAAGAC-3	5-TGAGGGAGATGCTCAGTGTT-3

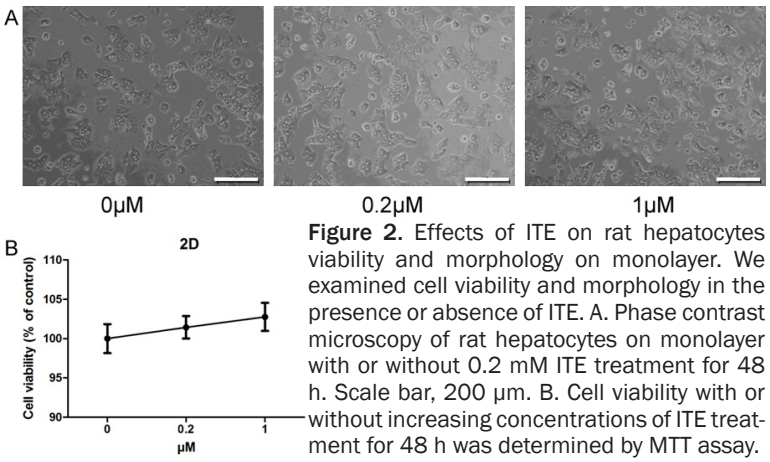

Figure 2. Effects of ITE on rat hepatocytes viability and morphology on monolayer. We examined cell viability and morphology in the presence or absence of ITE. A. Phase contrast microscopy of rat hepatocytes on monolayer with or without 0.2 mM ITE treatment for 48 h. Scale bar, 200 μm. B. Cell viability with or without increasing concentrations of ITE treatment for 48 h was determined by MTT assay.

Liver functions

Urea synthesis of rat hepatocytes was evaluated as typical liver functions. After 48 h of culturing, all medium was collected and stored at -80°C. The urea concentration was measured with the urea assay kit (DIUR-500, BioAssay systems, Hayward, CA). All results were analyzed with CurveExpert 1.3 software and fitted with Logistic Model with $r^2 > 0.99$. All these values were normalized with the cell number in the supernatant on the same day.

Real-time PCR

After incubating with/without ITE in those three different culture conditions above for 48 h, microspheres were dissolved in 55 mM sodium citrate. After washing with PBS twice, RNA was extracted using the RNeasy Mini kit (15596026, Qiagen). cDNA was synthesized using oligo-primers and a reverse transcription kit (037A; TAKARA, Shiga, Japan). The Bio-Rad universal SYBR Supermix (72-5121, Bio-Rad, Hercules, CA) was used to perform real-time quantitative PCR assays on a Bio-Rad Cycler (C1000, Bio-Rad, Hercules, CA) with various sequences (**Table 1**).

Statistical analysis

Statistical analysis was performed using the Student's t-test and one-way ANOVA with SPSS for Windows version 20.0 (Chicago, IL, United States). Data from representative experiments were presented as means ± standard deviation (SD). Differences were considered statistically significant with P values < 0.05. Experiments were repeated at least three times in triplicate for each condition.

Results

Effect of ITE on rat hepatocytes viability

In order to explore the potential toxicity of ITE on rat hepatocytes, we examined cell viability and morphology in the presence or absence of ITE (**Figure 2**). Phase contrast microscopy showed that a concentration of up to 1.0 μM

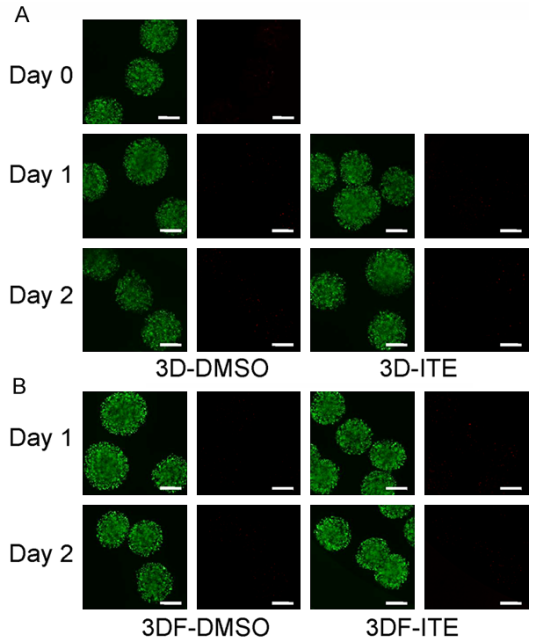

Figure 3. Effects of ITE on rat hepatocytes viability in microspheres. Phase and fluorescence microscopy images of rat hepatocytes in microspheres with and without 1 mM ITE treatment for 24 h and 48 h. Scale bar, 500 μm. Living cells were stained in green (CM-FDA), whereas dead cells were stained in red using PI (propidium iodide).

ITE treatment for 48 h did not have discernable effects on the general morphology of rat hepatocytes on monolayer (**Figure 2A**). The MTT assay showed ITE at a concentration of up to 1.0 μM ITE treatment for 48 h did not affect the growth of rat hepatocytes on monolayer (**Figure 2B**). Meanwhile the morphology of rat hepatocytes cultured in alginate microspheres was not affected by ITE, viability measured by FDA/PI staining indicated that ITE (1.0 μM) showed minimally toxic to the growth of rat hepatocytes in alginate microspheres (**Figure 3A**).

Liver-specific gene expression in rat hepatocytes

We evaluated the effect of ITE on transcription of several metabolism associated genes including CYP450 phase I and phase II enzymes as well as nuclear receptors. We focused on comparing the expression profiles of rat hepatocytes in ITE culturing versus normal culturing on monolayer, microspheres in static condition and in a fluidized bed bioreactor (**Figure 4**). Our results showed that transcription levels of most CYP450-related genes were significantly elevated by ITE. Furthermore under 3DF condition, the rat hepatocytes in microsphere exhibited higher transcription levels than those in 2D and 3D condition.

Metabolic functions of rat hepatocytes

In order to assess the biotransformation capacity of rat hepatocytes in the presence of ITE, we examined CYP450 activity of rat hepatocytes (**Figure 5**). CYP1A2 and CYP3A4 activity levels were both increased in rat hepatocytes cultured with ITE. Interestingly CYP3A4 exhibited higher improvement in its metabolic activity in those culture conditions (**Figure 5B** and **5E**). In summary the enhanced CYP450s activity levels indicated a higher detoxification capacity in liver cells maintained in our new culture condition.

Ureogenesis of rat hepatocytes

In order to evaluate the liver function with ITE treatment, urea synthesis was measured with the urea assay kit. It showed ureogenesis of rat hepatocytes were improved by ITE in our three culture conditions (**Figure 5C** and **5F**). However, this advancement seemed less than those of CYP1A2 and CYP3A4, indicating there may be a higher detoxification capacity of the liver cells other than biological synthesis upon ITE treatment.

Discussion

In the present study, we cultured rat hepatocytes with ITE on monolayer and microspheres in static condition and in a fluidized bed bioreactor. We compared functions of rat hepatocyte with/without ITE by characterizing its hepatic functionality, metabolism, and proliferative capacity. Here we provided evidences that ITE treatment increased functions of rat hepatocytes. This is based on the following observations: (1) maintenance of the proliferation capacity; (2) an increase in expression levels of several important hepatic genes involved in detoxification; and (3) an improvement in CYP-1A2 and CYP3A4 activity.

The CYP450 superfamily is involved in metabolism of drugs, chemicals and endogenous substrates. Among CYP450 family, CYP1A2 and CYP3A4 are frequently investigated in human

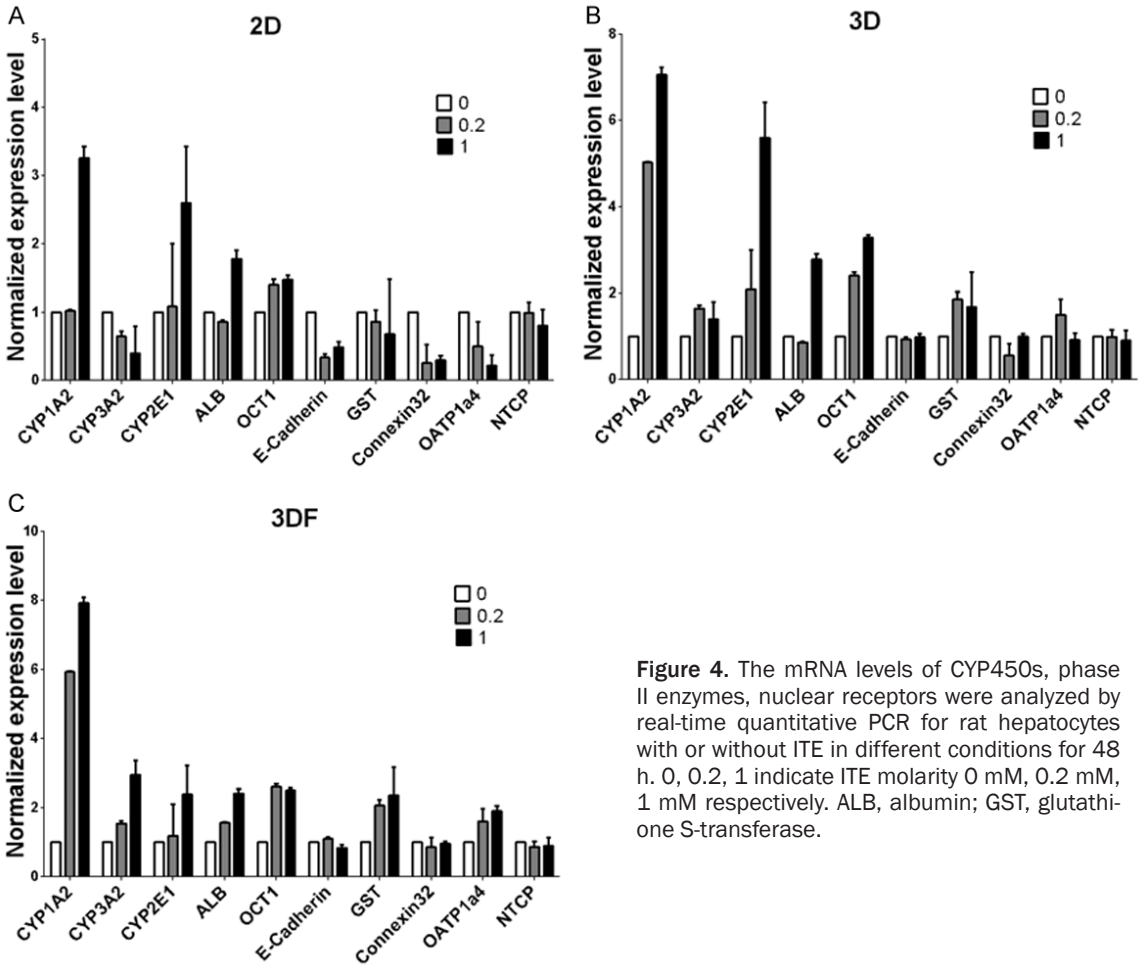

Figure 4. The mRNA levels of CYP450s, phase II enzymes, nuclear receptors were analyzed by real-time quantitative PCR for rat hepatocytes with or without ITE in different conditions for 48 h. 0, 0.2, 1 indicate ITE molarity 0 mM, 0.2 mM, 1 mM respectively. ALB, albumin; GST, glutathione S-transferase.

liver since they play essential roles in drug clearance [15-18]. Numerous studies have demonstrated that AhR regulated all CYP450 enzymes that were also induced by aromatic hydrocarbons such as TCDD and 3-methylcholanthrene (MC) [19]. AhR is a cytosolic transcription factor that is normally inactive and bound to several co-chaperones. Upon binding to its ligands such as ITE, the chaperones dissociate resulting in AhR translocating into the nucleus. This leads to its formation of a heterodimer with the closely-related Arnt nuclear protein. Accordingly, the AhR/ARNT complex can alter the transcription of the CYP1 enzymes and thereby increasing CYP450 activity [20]. In agreement with previous observations in other cell lines, our results showed that ITE could enhance the activity of CYP450 enzymes with no discernable toxic effect on rat hepatocytes.

CYP450 activities and the gene expressions were higher in a fluidized bed bioreactor than those of monolayer and microspheres in static condition, which was consistent with previous studies [21, 22], suggesting that a fluidized bed bioreactor was of benefit to the hepatocytes functions in future clinical applications.

In this study, we determined effects of ITE on rat hepatocytes cultured on monolayer, microspheres in static condition and a fluidized bed bioreactor. Our data showed that the addition of ITE to hepatocytes in our three culturing conditions above improved metabolic activities of major cytochrome P450 enzymes and metabolic gene expressions of rat hepatocytes. Altogether, the improvement of rat hepatocytes functions indicated ITE could have impact on the in vitro application of these cells in toxicology assays and drug screening.

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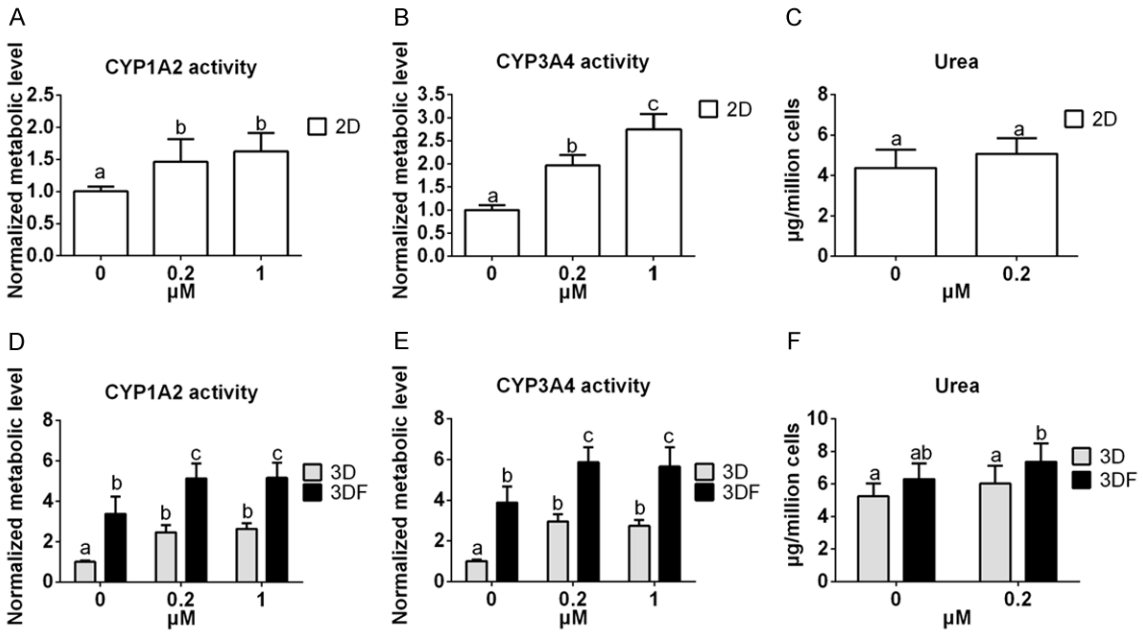

Figure 5. Determination of CYP450 enzymatic activities and ureogenesis of rat hepatocytes cultured on monolayer, as microspheres and in a fluidized bioreactor. The activities of phase I enzymes (CYP1A2 and CYP3A4) were measured via fluorometric substrates in different culture conditions. A, B. Enzymatic activities of phase I enzymes (CYP1A2 and CYP3A4) with different concentrations of ITE for 48 h on monolayer. C. Ureogenesis of rat hepatocytes with different concentrations of ITE for 48 h on monolayer were measured with the urea assay kit. D, E. Enzymatic activities of phase I enzymes (CYP1A2 and CYP3A4) with different concentrations of ITE for 48 h as microspheres and in a fluidized bioreactor. F. Ureogenesis of rat hepatocytes with different concentrations of ITE for 48 h as microspheres and in a fluidized bioreactor were measured with the urea assay kit. All data were normalized to the activity of rat hepatocytes cultured in a dish. Columns labeled with the same letter indicate conditions not statistically significant.

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Disclosure of conflict of interest

None.

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