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Gel entrapment culture of rat hepatocytes for investigation of tetracycline-induced toxicity

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ABSTRACT

This paper aimed to explore three-dimensionally cultured hepatocytes for testing drug-induced nonalcoholic steatohepatitis. Gel entrapped rat hepatocytes were applied for investigation of the tetracycline-induced steatohepatitis, while hepatocyte monolayer was set as a control. The toxic responses of hepatocytes were systematically evaluated by measuring cell viability, liver-specific function, lipid accumulation, oxidative stress, adenosine triphosphate content and mitochondrial membrane potential. The results suggested that gel entrapped hepatocytes showed cell death after 96 h of tetracycline treatment at 25 μM which is equivalent to toxic serum concentration in rats, while hepatocyte monolayer showed cell death at a high dose of 200 μM. The concentration-dependent accumulation of lipid as well as mitochondrial damage were regarded as two early events for tetracycline hepatotoxicity in gel entrapment culture due to their detectability ahead of subsequent increase of oxidative stress and a final cell death. Furthermore, the potent protection of fenofibrate and fructose-1,6-diphosphate were evidenced in only gel entrapment culture with higher expressions on the genes related to β-oxidation than hepatocyte monolayer, suggesting the mediation of lipid metabolism and mitochondrial damage in tetracycline toxicity. Overall, gel entrapped hepatocytes in three-dimension reflected more of the tetracycline toxicity *in vivo* than hepatocyte monolayer and thus was suggested as a more relevant system for evaluating steatogenic drugs.

Introduction

Nonalcoholic steatohepatitis (NASH) is combination of lipid accumulation in liver and associated liver lesions, such as lipid peroxidation and cell necrosis/apoptosis. Tetracycline is a well-known antibiotic that induces NASH in human and rodents since the first description in 1951 (Lepper et al., 1951).

Tetracycline-induced NASH has been extensively reported in studies *in vivo* (Mukherjee and Mukherjee, 1969; Mikhail et al., 1980; Freneaux et al., 1988; Labbe et al., 1991; Yin et al., 2006). Inhibition on mitochondrial β-oxidation of fatty acids largely determined tetracycline-induced steatohepatitis in mice, which had been confirmed by radioactive assay (Freneaux et al., 1988) and gene microarray on β-oxidation rate (Yin et al., 2006). Besides, tetracycline was suggested to block tricarboxylic acid cycle and respiratory chain in mitochondria in rats (Fromenty and Pessayre, 1995). Triglyceride accumulation and lipid peroxidation had also been detected, following by increase of serum alanine and aspartate aminotransferases in rodents (Freneaux et al., 1988; Letteron et al., 1996; Kikkawa et al., 2006) and human (Wruble and Cummins, 1965; Robinson and Rywlin, 1970).

Relatively, tetracycline toxicity has been partly evidenced basing on a few *in vitro* studies using hepatocyte monolayer. The reduced metabolism of fatty acid and lipid accumulation were observed in tetracycline-treated hepatocytes from either rats (de Longueville et al., 2003) or dogs (Amacher and Martin, 1997) while oxidative stress was demonstrated in rat hepatocytes (Yamamoto et al., 2005) as well as in human hepatocytes (Xu et al., 2008). In addition, decrease of mitochondrial membrane potential was detected in human hepatocytes by treatment of tetracycline (Xu et al., 2008). Thus, these results by monolayer culture concerned lipid accumulation, oxidative stress or mitochondrial lesion which only reflected a part of cellular alternations without systematic description.

However, the ongoing processes of tetracycline toxicity were differentially evidenced between hepatocyte monolayer *in vitro* and animals or human *in vivo*. It was noticed that the detectable toxic concentration of tetracycline in monolayer culture of hepatocytes were mostly above 100 μM (de Longueville et al., 2003), while the toxic serum concentrations of tetracycline were around 12.4 μg/ml (equivalent to 27 μM) after a single administration of 2000 mg/kg for

Abbreviations: ATP, adenosine triphosphate; Crat, carnitine acyltransferase; CYP, cytochrome; Dci, dodecenoyl-CoA delta isomerase; DTNB, 5,5-dithiobis(2-nitrobenzoic acid); GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GSH, glutathione; MDA, malondialdehyde; MTT, methyl thiazolyl tetrazolium; NASH, nonalcoholic steatohepatitis; PBS, phosphate buffer solution; PPAR, peroxisome proliferater activated receptor; RFU, relative fluorescence units; ROS, reactive oxygen species; RT-PCR, reverse transcription-polymerase chain reaction; TBA, thiobarbituric acid.

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6 h in rats (Kikkawa et al., 2006) and 9.3 μg/ml (equivalent to 21 μM) in human (Xu et al., 2008). Moreover, treatment by tetracycline higher than 300 μM triggered only a slight accumulation of lipid/triglyceride (Amacher and Martin, 1997; Xu et al., 2008) and no depletion of GSH (Xu et al., 2008) before causing severe cell death in hepatocyte monolayers. This was not in agreement with the observation *in vivo* that the tetracycline-induced lipid accumulation and anti-oxidant depletion occurred ahead of cell death (Letteron et al., 1996). Alternatively, no time-dependent accumulation of triglyceride was noticed according to the unchanged lipid content at 24 h and 48 h in hepatocyte monolayer (Amacher and Martin, 1997), which was in opposite to the time-dependent accumulation of triglyceride *in vivo* (Letteron et al., 1996). In this respect, we premised that hepatocyte monolayer commonly insensitive to drug hepatotoxicity was caused by their lowly sustained liver-specific functions.

Previously, we demonstrated that collagen gel entrapped hepatocytes with highly expressed metabolic enzyme activities showed high susceptibility to CYP 450-meditated drug hepatotoxicity (Shen et al., 2006, 2007). It is unknown whether gel entrapment of hepatocytes could be a more effective system for evaluation of drug-induced NASH. Hence, this paper explored such applicability by systematically examining cellular alterations including mitochondrial damage, lipid accumulation and oxidative stress when exposed to tetracycline in gel entrapped rat hepatocytes and further addressed the involved toxic mechanism by comparison with hepatocyte monolayer.

Materials and methods

Chemicals. Tetracycline was from Bio Basic Inc. (Markham Ontario, Canada). Williams' E basal medium and collagenase (type IV) were purchased from Gibco (Gaithersburg, USA). MTT, L-glutamine, penicillin and streptomycin were purchased from Amresco Inc. (Solon, Ohio, USA). Fenofibrate, 5,5-dithiobis(2-nitrobenzoic acid) (DTNB), Nile red, insulin, dexamethasone and glucagon were purchased from Sigma (St. Louis, MO, USA). Collagen (type I) was purchased from BD Biosciences (Bedford, MA, USA). The rat albumin ELISA quantitation kit was purchased from BETHYL Laboratories, Inc. (Montgomery, TX, USA). Fetal bovine serum was obtained from Hangzhou Sijiqing Biological Eng. Material Co., Ltd. (Hangzhou, China). Rhodamine 123, dihydrorhodamine 123, RNA isolation kit, first strand cDNA synthesis kit and PCR reagents were purchased from Invitrogen (Karlsruhe, Germany). Tetraethoxypropane was a gift from Xinjing Co., Ltd. (Wuhan, China). The primers were synthesized by SBS Genetech Co., Ltd (Shanghai, China). Triglyceride assay kit was from Saike Biological Technology Co., Ltd. (Ningbo, China). The remaining chemicals were obtained from local chemical suppliers and were all of reagent grade.

Hepatocyte cultures. All animal procedures were carried out in accordance with the Guide for the Care and Use of Laboratory Animals by the United States National Institutes of Health. Hepatocytes were isolated from male Sprague–Dawley rats (weighing 200–250 g) by the two-step collagenase perfusion method as previously described (Wu et al., 2005). Cell viability was assessed by Trypan blue exclusion and hepatocytes with a viability of greater than 85% were used.

Hepatocytes were cultured in Williams' E medium supplemented with L-glutamine 2 mM, penicillin 100 U/ml, streptomycin 100 μg/ml, dexamethasone 1 μM, insulin 0.2 U/ml, glucagon 4 ng/ml and 5% (v/v) fetal bovine serum.

For the monolayer culture, each well of 24-well plates was precoated with 0.16 mg/l of collagen (type I). Freshly isolated rat hepatocytes were seeded at a density of 2×10^5 cells/well. The cells were cultured at 37 °C in a humidified atmosphere containing 5% CO_2.

For the gel entrapment culture, freshly isolated hepatocytes were mixed with the collagen solution and loaded into hollow fibers (polysulfone, 100 kDa molecular weight cut-off, Shanghai, China) by injection as described before (Wu et al., 2005). Briefly, hepatocytes were inoculated into a 3:1 (v/v) mixture of type I collagen (2.75 mg/ml) and four-fold concentrated Williams' E medium at pH 7.4. Hepatocyte suspension at a density of 1×10^6 cells/ml was loaded into the lumen of hollow fibers and maintained in a 5% CO_2 incubator for collagen gelation. Ten minutes later, hollow fibers were put into 6 cm culture dishes full of 8 ml prewarmed culture medium and put into a 5% CO_2 incubator for hepatocyte cultures.

Exposure of hepatocytes to tetracycline. At 4 h after plating or entrapment, hepatocytes in monolayer or gel entrapment were washed with phosphate buffer solution (PBS) and then treated with tetracycline (25, 50, 75, 100 and 200 μM) for 48 h or 96 h, respectively. The culture medium was changed everyday before the assays were conducted.

Effect of fenofibrate and fructose-1,6-diphosphate on tetracycline-induced hepatotoxicity. At 4 h after plating or entrapment, hepatocytes in monolayer or gel entrapment were washed with phosphate buffer solution (PBS) and then treated with tetracycline, fenofibrate and tetracycline together with fenofibrate for 48 h. Alternately, the hepatocytes were treated by tetracycline, fructose-1,6-diphosphate and their combinational usage for 48 h, respectively.

Cell viability assay. The MTT assay was used to evaluate the viability of hepatocyte in monolayer and gel entrapment. Briefly, the gel entrapped hepatocytes were extruded from the hollow fibers with a 5 ml syringe and immersed in 0.65 ml of the MTT-PBS (1.15 mg/ml) in 24-well plates followed by incubation at 37 °C for 3 h. After removing the MTT solution, 1.5 ml of isopropanol was added to the cells. After agitation for 1 h, the absorbance of the solution containing the extracts was read at 570 nm on a spectrophotometer as previously described (Wang et al., 1996). The same procedure was used for determination of the viability of hepatocytes in monolayer cultures.

Glutathione (GSH) assay. Hepatocytes in monolayer cultures were rinsed with PBS and scraped off from the wells with a rubber policeman. Gel entrapped cells were washed with PBS, released from the hollow fibers as described above and then suspended in 100 μl of PBS. After collection, the hepatocytes were sonicated at 40 kHz and 900 W for 10 s and centrifuged at 16,000 ×*g* to precipitate cellular fragments. The GSH content in cell supernatants was determined by DTNB assay as previously described (Nieusma et al., 1998).

Reactive oxygen species (ROS) assay. Accumulation of intracellular ROS was assayed by dihydrorhodamine 123. In details, hepatocytes (2×10^5 cells) in both monolayer and gel entrapment were incubated in PBS with 10 μM dihydrorhodamine 123 for 30 min at 37 °C. Then, the fluorescence was determined by a fluorescence microplate reader (Tecan, Germany) with excitation at 488 nm and emission at 535 nm (Qu et al., 2001).

Lipid accumulation in hepatocytes by Nile red staining. The lipid accumulation in cultured hepatocytes was determined fluorimetrically using Nile red, a vital lipophilic dye used to label lipid droplets in the cytosol. The gel entrapped hepatocytes were firstly extruded from the hollow fibers with a 5 ml syringe, whereas collagen gel without cells was set as a blank for gel entrapped hepatocytes. Both hepatocyte monolayer and gel entrapped hepatocytes were washed with PBS and then incubated for 30 min with Nile red solution at a final concentration of 3 μM in PBS at 37 °C. Hepatocytes were washed thereafter with PBS and detected in a fluorescence microplate reader (Tecan, Germany) at excitation 580 nm and emission 630 nm (Donato et al., 2006).