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A Novel 3D Liver Organoid System for Elucidation of Hepatic Glucose Metabolism

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Abstract

Hepatic glucose metabolism is a key player in diseases such as obesity and diabetes as well as in antihyperglycemic drugs screening. Hepatocytes culture in two-dimensional configurations is limited in vitro model for hepatocytes to function properly, while truly practical platforms to perform three-dimensional (3D) culture are unavailable. In this work, we present a practical organoid culture method of hepatocytes for elucidation of glucose metabolism under nominal and stress conditions. Employing this new method of culturing cells within a hollow fiber reactor, hepatocytes were observed to self-assemble into 3D spherical organoids with preservation of tight junctions and display increased liver-specific functions. Compared to both monolayer culture and sandwich culture, the hepatocyte organoids displayed higher intracellular glycogen content, glucose consumption, and gluconeogenesis and approached the in vivo values, as also confirmed by gene expression of key enzymes. Moreover, hepatocyte organoids demonstrated more realistic sensitivity to hormonal challenges with insulin, glucagon, and dexamethasone. Finally, the exposure to high glucose demonstrated toxicities including alteration of mitochondrial membrane potential, lipid accumulation, and reactive oxygen species formation, similar to the in vivo responses, which was not captured by monolayer cultures. Collectively, hepatocyte organoids mimicked the in vivo functions better than hepatocyte monolayer and sandwich cultures, suggesting suitability for applications such as antihyperglycemic drugs screening.

Keywords

rat hepatocytes; 3D organoid culture; hollow fiber bioreactor; glucose metabolism

Introduction

Liver is one of the most important organs in maintaining normal physiological functions of body. It has often been considered as a complicated bioreactor for managing carbohydrate,

lipid, and protein metabolism in the body (Soboll, 1995). For example, the hepatic metabolism can regulate blood glucose concentrations within a narrow range (3.9–6.1 mM) via either preventing hyperglycemia in the fed state or avoiding hypoglycemia in fasting state under the appropriate regulation of hormones including glucagon and insulin (Klover and Mooney, 2004). This is achieved by rapid transformation of glucose in plasma to hepatic glycogen in presence of insulin (postprandial response), and reverse in presence of glucagon (fasting response). With the rise of obesity, diabetes, and other nutritional/metabolic diseases, the role of the liver has increasingly come into focus in pathophysiologic studies, especially for investigating the disorders of liver metabolism (Qureshi and Abrams, 2007) and screening effective drug treatment for such metabolic disorders (Tolman et al., 2009; Yki-Jarvinen, 2009). Hence, liver can be an extremely important organ for not only physiological and pathophysiologic studies but also for evaluation of drugs in treatment of metabolism disorders like diabetes and obesity.

Primary hepatocyte cultures are valuable models for elucidating cellular and molecular phenomena occurring in liver mechanistically (Nahmias et al., 2007), and can partially substitute for animal testing. Among the hepatocyte culture models in vitro, monolayer culture is the most conventional one, for ease of set-up and manipulation, and thus still in active use for drug screening metabolic studies (Dash et al., 2009; Lauer et al., 2009). The collagen sandwich culture system is widely acknowledged as a better configuration (Tuschl et al., 2009), which was found more suitable for measurements of efflux transport or biliary excretion (Marion et al., 2007) compared with the other culture systems. Still, for study of hepatic glucose metabolism in vitro, hepatocyte monolayer is still the most common culture configuration (Klein et al., 2002; Mashek and Grummer, 2003; Raman et al., 2004). Nevertheless, hepatocytes in monolayer configuration are well known for rapid loss of liver-specific functions (Brandon et al., 2003) do not display significant gluconeogenesis after 2 days of culture (Yamada et al., 1980) and even change their metabolic pattern towards that of permanent cell lines after 3 days of culture (Bissell et al., 1978). The sandwich culture is less applied for glucose metabolism studies and limited reports employing this approach also suggested that cells do not respond fully to insulin stimulation (Hansson et al., 2004).

To overcome the shortcomings of two-dimensional (2D) culture, the three-dimensional (3D) cultured hepatocytes have been developed and used as liver specific functions are better preserved compared to monolayer (Wu et al., 1999), including higher sensitivity to drug-induced hepatotoxicity (Meng, 2010). Using gel entrapment culture (i.e., gel disk culture), hepatocytes express higher glucose metabolism activities in glucose synthesis and gluconeogenesis in comparison with monolayer culture (Wen et al., 2009). However, the key glucose-related metabolic functions, including phosphoenolpyruvate carboxykinase (PEPCK) as well as CCAAT/enhancer-binding protein α (C/EBP) declined sharply within 24 h of culture and it has been suggested that 3D culture condition needs further optimization (Wen et al., 2009). The impact of other 3D culture in the context of glucose metabolism, response to hormone and high glucose stimulation as well as metabolic diseases in general has been little investigated.

In this study, we constructed a hollow fiber based organoid culture of hepatocytes and systematically evaluated the glucose metabolism to assess its capability for screening diabetes drugs. Glucose metabolism, enzyme activity, and gene expressions of organoid cultures were compared with traditional monolayer culture as well as sandwich culture configurations. Hormonal response and hyperglycemia-induced pathology were also examined for evaluation of 3D organoid bioreactor platform.

Materials and Methods

Primary Hepatocyte Isolation and Culture

Male Sprague-Dawley rats (Zhejiang Academy of Medical Sciences, China) weighing 200–300 g and fed ad libitum were used for all studies. Hepatocytes were isolated 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 >85% were used in the studies.

For monolayer culture, freshly isolated hepatocytes were seeded at a density of 5×10^4 cells/cm², in the collagen-coated (0.3 mg/mL collagen type I) culture plates (Corning Costar, Cambridge, MA) for static culture. For sandwich culture, hepatocytes were firstly seeded in gelled collagen coated plates at a density of 5×10^4 cells/cm². After 24 h of incubation at 37°C, neutralized collagen solution (0.01 mL/cm², 1.5 mg/mL, pH 7.4) was added to each well of the plates. Cultures were incubated for 30 min at 37°C in a humidified incubator to gelate the collagen before the addition of culture medium. For the organoid culture, freshly harvested hepatocytes with a density of 1×10^6 cells/mL were inoculated into a 3:1 (v/v) mixture of collagen type I (0.3 mg/mL) and fourfold concentrated DMEM medium (pH 7.4). Then the whole mixture was loaded into polysulphone-g-poly (ethylene glycol) (PSf-g-PEG) hollow fiber membrane by injection and incubated in a “groove culture plate” covered with 1.5 mL standard culture medium in each groove well. The groove culture plate was custom-made by Zhejiang Gongdong Medical Technology Co., Ltd (Zhejiang, China), according to our issued patent (Patent No. CN 200610053989.8). It was of similar configuration and material (polystyrene) as Costar culture plates except that the multi wells was replaced by an array of 12-paralled channels (70 × 5 × 10 mm, L × W × H, separated by barriers with 3 mm in width). The PSf-g-PEG hollow fibers previously fabricated ourselves had inner/outer diameter of 0.6/1.0 mm and membrane porosity of 80% (Shen et al., 2010). The pore volume showed a Gaussian distribution between 10 and 1,000 nm with an average pore radius of 90.8 nm as measured by automatic mercury porosimeter (AutoPore IV 9500, Norcross, GA).

Each hepatocyte culture was conducted in a standard medium consisting phenol red-free low glucose Dulbecco's Modified Eagle Medium (Gibco, Gaithersburg, MD) supplemented with 5 mM glucose, 1% BSA (bovine serum albumin), 2 mM L-glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin, 100 nM dexamethasone, 10 nM insulin, 1 nM glucagon (all from Sigma, St. Louis, MO).

Morphological Analysis

Hepatocyte morphology was examined with an inverted microscope (XD-101, Olympus, Japan) and cell ultrastructure was investigated by scanning electron microscopy (SEM) and transmission electron microscopy (TEM). The hepatocytes organoids were extruded from hollow fibers by injection with a 5-mL syringe full of PBS solution, and then immediately collected and fixed overnight in 1 mL of 4% (v/v) glutaraldehyde at 4°C before SEM (Philips XL-30 ESEM, Eindhoven, Netherlands) and TEM (JEM-1200EX, JEOL, Japan) observation.

Assays of Liver-Specific Functions

Culture medium was collected at the indicated time point for both urea and albumin secretion analysis. Urea assays were performed using a Urea Nitrogen Kit. Rat albumin concentrations were determined by enzyme-linked immunosorbent assay (ELISA), as previously described (Shen et al., 2006). For absolute value, all the data were normalized to

the protein content of the hepatocytes as measured with bicinchoninic acid (BCA) (BCA Protein Assay kit, Thermo Scientific Pierce, Rockford, IL).

Glucose Consumption, Glycogen Content and Glucose Synthetic Ability

For determination of glucose consumption, the glucose concentration of culture medium collected at the indicated time was determined using a colorimetric glucose assay kit according to the manufacture's instructions (Sigma-Aldrich, St. Louis, MO). The glucose consumption of the hepatocytes was calculated by the glucose concentrations of blank wells subtracting the remaining glucose in cell-plated wells.

For glycogen content assay, hepatocytes in both monolayer and sandwich cultures were rinsed with PBS and scraped off from the wells with a rubber policeman while organoid cells were released from the hollow fibers by syringe injection and washed with PBS before suspended in 100 μ l of 0.2 M sodium acetate buffer. All the cell samples were kept in -70°C before assay. Intracellular glycogen content was determined by treating the cell lysates with amyloglucosidase (EC 3.2.1.3) at pH 4.8 and 40°C for 2 h according to previous work with some modifications (Gomez-Lechon et al., 1996).

For glucose synthetic ability assay, the hepatocyte cultures were washed with PBS for three times before incubation in glucose-free buffer (117.6 mM NaCl, 5.4 mM KCl, 0.82 mM Mg_2SO_4 , 1.5 mM KH_2PO_4 , 20 mM Hepes, 9 mM NaHCO_3 , 0.1% w/v BSA, and 2.25 mM CaCl_2 , pH 7.4). The glycogenolysis rate was expressed by glucose generation within 1 h of incubation in the glucose-free buffer with or without glucagon, while the rates of gluconeogenesis of the hepatocytes were determined by glucose generation within 1 h incubation in the same glucose-free buffer with the presence of pyruvate (10 mM) or glycerol (10 mM) (Loven et al., 2005).

In order to avoid different amount of glucose residue in the different culture models, cell-free cultures were performed as a control for glucose assay.

Gene Expression Analysis

Total RNA was extracted from freshly harvested cells or cultured cells with Trizol Reagent (Sbsbio, Shanghai, China). Double-stranded cDNA was synthesized from 1 μ g of total RNA using a hexamer primer. Obtained cDNA was used as a template for in vitro transcription using the commercial kit purchased from Takara (Otsu Shiga, Japan) as described before (Wilkening and Bader, 2003) and amplified by the polymerase chain reaction (PCR). The expression of the gene for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in hepatocytes was used as a control (Yin et al., 2006). All the sequences of the primers are listed in Table I.

PEPCK Activity Determination

PEPCK activity was determined as described by Petrescu et al. (1979). At the indicated culture time, hepatocytes in each culture were collected as mentioned above and then were homogenized at 4°C in PBS by sonication. Oxaloacetate formed during the enzyme reaction was reduced with malate dehydrogenase and NADH. The concomitant loss of NADH absorbance was monitored at 340 nm. The reaction was allowed to proceed for 5 min at 23°C . Change in absorbance versus time was converted to μmol of product formed per min by means of the molar coefficient of absorbance of NADH ($6.22 \text{ cm}^2/\mu\text{mol}$ at 340 nm). PEPCK activity was expressed as μmol of oxaloacetate formed per mg protein per minute.

Hormonal Response Assay

For hormonal response assay, hepatocytes in each culture model were preincubated in standard serum-free culture medium for 2 days, followed by culture in hormone-free medium for 3 h. The cells were incubated for an additional 6 h in the presence or absence of hormones and glucose as stated in the text and subjected to intracellular glycogen assay. The corresponding concentration of insulin, glucagon, and dexamethasone were 10, 1, and 100 nM, respectively. Hepatocyte cultures without any additions were included as the control groups.

High Glucose Response Assay

For different glucose concentration culture, the hepatocytes in monolayer and organoid cultures were respectively incubated in different concentration of glucose at the beginning of cell culture. The culture medium was changed every other day. After 6 days of glucose treatment, hepatocytes were washed, collected and subjected to analysis of cell viability (MTT) (Wang et al., 1996), mitochondrial membrane potential (MMP) (Masubuchi et al., 2006), reactive oxygen species (ROS) (Qu et al., 2001), malondialdehyde (MDA) (Londero and Lo Greco, 1996), Nile red staining (Donato et al., 2006), and intracellular triglyceride (TG) content (El-Assal et al., 2004) assays.

Statistics

All the experiments were tested with three replicates, with cells obtained from three different rats, if not indicated otherwise. Values reported are means $\pm$ SD. Comparisons between multiple groups were performed with ANOVA and Turkey post hoc analysis procedures. *P*-values < 0.05 were considered statistically significant.

Results

Hepatocyte Performance in Organoid Culture

Morphology of hepatocytes in the hollow fibers was examined under both bright field microscope (Fig. 1A–C) and electronic microscopy (SEM and TEM) (Fig. 1D–F). After 1 day of culture, hepatocytes gradually contacted with each other and formed 3D and columnar-shaped organoids along the whole hollow fibers (Fig. 1A–C). The average diameter of the formed organoids was determined to be about 100 μ m by examination of at least 60 visual fields using a photomicrograph. The organoids were wrapped by a thin layer of collagen fibers on the outer surface (Fig. 1E). The hepatocytes in organoids exhibited polygonal shapes, possessing round shape of nuclei (N), extensive cell–cell contact, numerous mitochondria (M) and microvilli (m). Abundant bile-canalculus (bc) like structures were observed between hepatocytes (Fig. 1F).

For examination of liver-specific functions in each culture, the culture medium was respectively collected at 2, 4, 8, and 14 days in each culture for urea genesis and albumin synthesis analysis. The result displayed that hepatocytes organoids maintained much higher liver-specific functions than either monolayer or sandwich culture (Table II). Hepatocytes in organoid culture could maintain albumin and urea synthetic functions for at least 2 weeks, while hepatocytes in monolayer culture could be moderately maintained for only 4 days (Table II). In this respect, 2- or 4-day culture was used to compare the glucose metabolism in the subsequent experiments.

Glucose Metabolism Activity

Each hepatocyte culture could respond to glucose exposure at a similar manner in that they produced glucose at low concentrations (5.5 mM) or consumed glucose at high level (>10

mM) (Fig. 2A). Nevertheless, consumption or production of glucose in hepatocyte organoid culture and sandwich culture was relatively higher than that in monolayer culture.

The intracellular glycogen content was respectively determined at 2 and 4 days of culture (Fig. 2B). In organoid culture, the cellular glycogen content was about 600 $\mu\text{mol/g}$ protein, which closely approached to the *in vivo* data of 792 $\mu\text{mol/g}$ protein (obtained from the fresh cells) and remained stable for at least 4 days. Hepatocytes in sandwich culture only had about the half glycogen content of hepatocyte organoids and hepatocyte monolayers were even worse.

Glucose synthetic ability was displayed by both glycogenolysis and gluconeogenesis rate. Both glycogenolysis and gluconeogenesis rates in hepatocytes organoids were almost comparable with the *in vivo* data and were much higher than hepatocyte monolayer and sandwich culture (Table III). The PEPCK activity was also analyzed in each culture at 2 days and 4 days, respectively (Fig. 3). As shown in this figure, hepatocytes showed higher PEPCK activity in organoid culture than in sandwich culture while hepatocytes in monolayer culture almost lost its gluconeogenesis at 4 days.

Differential Gene Expressions

The genes related to the glucose metabolism were selected and analyzed with PCR as reported in Table I with GADPH was selected as a housekeeping gene. The gene expressions were analyzed at 2 and 4 days, respectively (Fig. 4). All glucose metabolism-related genes were highly expressed in the freshly harvested hepatocytes. After the subsequent culture, the glucose metabolism related genes were dramatically down-regulated in monolayer as well as in sandwich culture after 2 days of culture and most of them were completely lost at 4 days of monolayer culture. Relatively, the hepatocyte organoids maintained gene expression of these enzymes, with the sole exception of GLUT 2.

Differential Hormonal Response

The effects of hormones on cellular glycogen accumulation were investigated in each culture configuration (Fig. 5). Although glycogen accumulation was well regulated by glucose in irrespective of culture models, the sensitivity was increased by insulin in organoid culture compared to monolayer/sandwich culture (Fig. 5A). The inhibitive regulation of glucagon on glycogen synthesis was observed only in organoid culture with no such function in either hepatocyte monolayer or sandwich culture (Fig. 5B). Similarly, hepatocytes in organoid culture showed a glycogen enhancement in response to dexamethasone while monolayer/sandwich culture had no significant changes of glycogen content under same hormone treatments (Fig. 5C).

High Glucose Response

The cellular alterations in the hepatocyte organoids induced by hyperglycemia, represented by oxidative stress, mitochondria dysfunctions, and lipid accumulation were evaluated and compared with responses of cells in monolayer, the standard culture method for drug testing. There was no significant cellular damage after 2–4 days of high glucose treatment (data not shown), so we prolonged the treatment until 6 days. Figure 6 displays these cellular responses to hyperglycemia at 6 days of treatment.

Lipid accumulation evaluated by both Nile red staining and TG determination was detected in hepatocyte organoids under glucose treatment. As shown in Figure 6A and B, intracellular TG content increased 35% by high glucose treatment at 44.8 mM compared with a normal glucose treatment at 5.5 mM. The Nile red staining displayed a similar trend under

hyperglycemia treatment in organoid culture. In stark contrast, there was no increase in lipid accumulation under monolayered hepatocytes.

Oxidative stress was evaluated by both ROS and MDA generation. ROS and MDA were increased in organoid cultures but not in monolayer culture as shown in Figure 6C and D. After treatment, 44.8 mM of glucose induced significant MDA accumulation by about 50% in organoids, while no obvious MDA changes were detected in monolayer culture.

The cell viability as indicated by MTT was not obviously altered in each culture while cellular MMP level declined significantly only in 3D organoid culture under high glucose treatment (Fig. 6E and F).

Discussion

A major function of the liver is maintenance of blood glucose homeostasis (Klover and Mooney, 2004). Consequently, it plays a critical role in glucose metabolism disorders and their treatments (Du et al., 2010). However, standard 2D in vitro systems such as monolayer and sandwich culture fail to fully replicate the response to hyperglycemia in vivo. Hence, the development of a practical 3D culture system, such as the hepatic bioreactor system presented in this work, which better mimics the physiological glucose metabolism of the liver in vivo is an urgently needed tool in pharmacological and pathological research.

In this study, we established a 3D organoid culture of primary hepatocytes for studying glucose-metabolism related research. Infusion of collagen/hepatocyte mixtures into hollow fibers led to self-assembly of these cells into 3D organoids. It was observed that in the organoid culture, hepatocytes displayed a cuboid shape, increased number of intercellular contacts and bile-canaliculi like structures. Consequently, hepatocyte organoid cultures displayed onefold higher albumin secretion and urea synthesis than the 2D hepatocyte culture configurations, in monolayer and sandwich culture (Table II).

In vivo, liver maintains glucose homeostasis by stimulating glycogenolysis and gluconeogenesis via activating PEPCK and G6Pase in fasted state as well as removing glucose via storing glycogen or metabolizing them through glycolysis in postprandial state (Klover and Mooney, 2004). Nevertheless, hepatocytes in monolayer and sandwich cultures failed to mimic the glucose metabolism of an intact liver, including glucose consumption, glycogenolysis, and gluconeogenesis (Table III, Figs. 2 and 3). As shown in Figure 4, expression of most of the glucose metabolism genes were lower in monolayer or sandwich culture and failed to capture the in vivo gene expression profile as previously stated (Boess et al., 2003). Rapid down-regulation of gluconeogenesis pathway under monolayer culture was observed in Figure 3 and Table III, as also reported in literature both at transcriptional (Baker et al., 2001) and phenotype levels (Yamada et al., 1980). By contrast, glucose consumption, glycogen storage, and glucose production and their gene expressions consistently all showed that organoid culture maintained higher glucose-related metabolic activity and approached the in vivo values, significantly better than either monolayer or sandwich culture. In particular, gluconeogenesis was very high in organoid cultures where as it essentially disappeared after 4 days in monolayer culture (Table III). These results were confirmed by both the enzymatic activity (Fig. 3) and transcriptional level (Fig. 4) of Pck1, a rate-controlling enzyme involved in gluconeogenesis. It should be mentioned that multiple genes are involved in glucose metabolism (i.e., glycogen accumulation). This could explain the in-corresponding correlation between glycogen maintenance (Fig. 2B) and Gys2 gene expression up-regulation (Fig. 4) of hepatocyte organoids at culture from 2 to 4 days. The situation is same for PEPCK, which was not mediated by only Pck1 (Beale et al., 2007) and

thus could illustrate the inconsistent correlation between a rapidly decreased PEPCK activity (Fig. 3) and a moderately decreased Pck1 gene expression (Fig. 4).

Moreover, hormones such as insulin, glucagon, and dexamethasone play a critical role in the liver during glucose homeostasis with a very high sensitivity at a low concentration (pM–nM level) in vivo (Allan and Titheradge, 1984; Noshiro et al., 1997). Although hepatocyte monolayer has been utilized for studying the hormonal response in vitro (Hermsdorf et al., 1999), the effects of hormone on glucose metabolism were not consistent with in vivo and even contradictory with varying incubation conditions (time, medium ingredient etc.) (Lopez et al., 1984; Yang et al., 2009). Our results (Fig. 5) and most of previous literature (Lopez et al., 1984; Yang et al., 2009) confirm the insensitivity of hepatocytes in monolayer and sandwich culture to the hormone stimulation. It was also been noted that, in the few reports where positive hormonal regulation was shown, a short preincubation time (18–24 h) and an extremely high concentration (1,000–100,000-folds higher than physiological level) of glucagon (Walker and Grindle, 1977) or dexamethasone (Salhanick et al., 1989) were needed to regulate glycogen in hepatocytes. The low sensitivities of hepatocyte monolayers were partially attributed to the loss of hepatic functions, such as decreased glucose metabolism enzymes (Walker and Grindle, 1977) and insulin-response receptor (Hansson et al., 2004). By stark contrast, the organoid culture with well-preserved glucose metabolism enzymes displayed good sensitivity to hormonal stimulation with insulin, glucagon, and dexamethasone at physiological concentration levels, and adequately reproduced the hormone stimulation response in vivo (Fig. 5).

Surprisingly, the sandwich culture which has been well accepted in literature for preserving liver-specific functions better than monolayer (Toritsuka et al., 2001) showed limited improvements in mimicking the glucose metabolism and no difference in replicating the hormonal stimulation compared with monolayers. Considering the additional complexities of this culture configuration and consequent increase in time and resource consumption, these results explain why in practice pharmaceutical industry does not abandon suspension or monolayer cultures in favor of the sandwich configuration. In our experience, the organoid culture was more convenient to prepare and operate and displayed improved function in all aspects that were measured.

As summarized in Table IV, hepatocyte organoid system was demonstrated to perform more in vivo like glucose metabolism up to 4 days and more sensitively respond to the physiological stimulation by hormones. Further, the hyperglycemia-induced pathogenic in vivo liver responses, such as mitochondria; lesions, lipid accumulation, and oxygen species generation (Du et al., 2010; Ling et al., 2003) were well mimicked by organoid culture during 6-day cultures. In contrast, the significant deviations of hepatic glucose metabolism from in vivo levels in monolayer are likely to explain why pharmacological efficacy of antidiabetic drugs are only observed under a drug dosage 100-folds higher than the concentrations in blood in vivo (Davies et al., 1999). Therefore, we conclude that long-term organoid culture is the ideal system to test the drug performance in vivo.

Conclusion

In summary, 3D hepatocyte organoid culture protocol established here demonstrated improved glucose metabolism, hormonal response as well as hyperglycemia induced cellular pathology of liver in vivo. This culture configuration is therefore a candidate as an improved, practical in vitro platform for physiological/pathological glucose metabolism studies and pharmacological investigations on anti-diabetes drugs and the like. Further, the hollow-fiber bioreactor also enables perfusion-culture of the cells, which could lead to a better performance of the organoids.

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References

- Allan EH, Titheradge MA. Effect of treatment of rats with dexamethasone in vivo on gluconeogenesis and metabolite compartmentation in subsequently isolated hepatocytes. *Biochem J.* 1984; 219(1): 117–123. [PubMed: 6721848]
- Baker TK, Carfagna MA, Gao H, Dow ER, Li Q, Searfoss GH, Ryan TP. Temporal gene expression analysis of monolayer cultured rat hepatocytes. *Chem Res Toxicol.* 2001; 14(9):1218–1231. [PubMed: 11559036]
- Beale EG, Harvey BJ, Forest C. PCK1 and PCK2 as candidate diabetes and obesity genes. *Cell Biochem Biophys.* 2007; 48(2–3):89–95. [PubMed: 17709878]
- Bissell DM, Levine GA, Bissell MJ. Glucose metabolism by adult hepatocytes in primary culture and by cell lines from rat liver. *Am J Physiol.* 1978; 234(3):C122–C130. [PubMed: 629333]
- Boess F, Kamber M, Romer S, Gasser R, Muller D, Albertini S, Suter L. Gene expression in two hepatic cell lines, cultured primary hepatocytes, and liver slices compared to the in vivo liver gene expression in rats: Possible implications for toxicogenomics use of in vitro systems. *Toxicol Sci.* 2003; 73(2):386–402. [PubMed: 12657743]
- Brandon EF, Raap CD, Meijerman I, Beijnen JH, Schellens JH. An update on in vitro test methods in human hepatic drug biotransformation research: Pros and cons. *Toxicol Appl Pharmacol.* 2003; 189(3):233–246. [PubMed: 12791308]
- Dash A, Inman W, Hoffmaster K, Sevidal S, Kelly J, Obach RS, Griffith LG, Tannenbaum SR. Liver tissue engineering in the evaluation of drug safety. *Expert Opin Drug Metab Toxicol.* 2009; 5(10): 1159–1174. [PubMed: 19637986]
- Davies GF, Khandelwal RL, Roesler WJ. Troglitazone inhibits expression of the phosphoenolpyruvate carboxykinase gene by an insulin-independent mechanism. *Biochim Biophys Acta.* 1999; 1451(1): 122–131. [PubMed: 10446394]
- Donato MT, Lahoz A, Jimenez N, Perez G, Serralta A, Mir J, Castell JV, Gomez-Lechon MJ. Potential impact of steatosis on cytochrome P450 enzymes of human hepatocytes isolated from fatty liver grafts. *Drug Metab Dispos.* 2006; 34(9):1556–1562. [PubMed: 16763015]
- Du D, Shi YH, Le GW. Oxidative stress induced by high-glucose diet in liver of C57BL/6J mice and its underlying mechanism. *Mol Biol Rep.* 2010; 37(8):3833–3839. [PubMed: 20217240]
- El-Assal O, Hong F, Kim WH, Radaeva S, Gao B. IL-6-deficient mice are susceptible to ethanol-induced hepatic steatosis: IL-6 protects against ethanol-induced oxidative stress and mitochondrial permeability transition in the liver. *Cell Mol Immunol.* 2004; 1(3):205–211. [PubMed: 16219169]
- Gomez-Lechon MJ, Ponsoda X, Castell JV. A microassay for measuring glycogen in 96-well-cultured cells. *Anal Biochem.* 1996; 236(2):296–301. [PubMed: 8660508]
- Hansson PK, Asztely AK, Clapham JC, Schreyer SA. Glucose and fatty acid metabolism in McA-RH7777 hepatoma cells vs. rat primary hepatocytes: Responsiveness to nutrient availability. *Biochim Biophys Acta.* 2004; 1684(1–3):54–62. [PubMed: 15450210]
- Hermisdorf T, Richter W, Dettmer D. Effects of dexamethasone and glucagon after long-term exposure on cyclic AMP phosphodiesterase 4 in cultured rat hepatocytes. *Cell Signal.* 1999; 11(9):685–690. [PubMed: 10530877]
- Ikezawa Y, Yamatani K, Ogawa A, Ohnuma H, Igarashi M, Daimon M, Manaka H, Sasaki H. Effects of glucagon on glycogenolysis and gluconeogenesis are region-specific in periportal and perivenous hepatocytes. *J Lab Clin Med.* 1998; 132(6):547–555. [PubMed: 9851746]
- Klein HH, Ullmann S, Drenckhan M, Grimmsmann T, Unthan-Fechner K, Probst I. Differential modulation of insulin actions by dexamethasone: Studies in primary cultures of adult rat hepatocytes. *J Hepatol.* 2002; 37(4):432–440. [PubMed: 12217595]
- Klover PJ, Mooney RA. Hepatocytes: Critical for glucose homeostasis. *Int J Biochem Cell Biol.* 2004; 36(5):753–758. [PubMed: 15061128]

- Lauer B, Tuschl G, Kling M, Mueller SO. Species-specific toxicity of diclofenac and troglitazone in primary human and rat hepatocytes. *Chem Biol Interact.* 2009; 179(1):17–24. [PubMed: 19022234]
- Ling PR, Mueller C, Smith RJ, Bistrian BR. Hyperglycemia induced by glucose infusion causes hepatic oxidative stress and systemic inflammation, but not STAT3 or MAP kinase activation in liver in rats. *Metabolism.* 2003; 52(7):868–874. [PubMed: 12870163]
- Londero D, Lo Greco P. Automated high-performance liquid chromatographic separation with spectrofluorometric detection of a malondialdehyde-thiobarbituric acid adduct in plasma. *J Chromatogr A.* 1996; 729(1–2):207–210. [PubMed: 9004941]
- Lopez MP, Gomez-Lechon MJ, Castell JV. Glycogen synthesis in serum-free cultured hepatocytes in response to insulin and dexamethasone. *In Vitro.* 1984; 20(12):923–931. [PubMed: 6397420]
- Loven AD, Olsen AK, Friis C, Andersen B. Phase I and II metabolism and carbohydrate metabolism in cultured cryopreserved porcine hepatocytes. *Chem Biol Interact.* 2005; 155(1–2):21–30. [PubMed: 15950209]
- Marion TL, Leslie EM, Brouwer KLR. Use of sandwich-cultured hepatocytes to evaluate impaired bile acid transport as a mechanism of drug-induced hepatotoxicity. *Mol Pharm.* 2007; 4(6):911–918. [PubMed: 17963355]
- Mashek DG, Grummer RR. Effects of long chain fatty acids on lipid and glucose metabolism in monolayer cultures of bovine hepatocytes. *J Dairy Sci.* 2003; 86(7):2390–2396. [PubMed: 12906057]
- Masubuchi Y, Kano S, Horie T. Mitochondrial permeability transition as a potential determinant of hepatotoxicity of antidiabetic thiazolidinediones. *Toxicology.* 2006; 222(3):233–239. [PubMed: 16621215]
- Meng Q. Three-dimensional culture of hepatocytes for prediction of drug-induced hepatotoxicity. *Expert Opin Drug Metab Toxicol.* 2010; 6(6):733–746. [PubMed: 20380484]
- Nahmias Y, Berthiaume F, Yarmush ML. Integration of technologies for hepatic tissue engineering. *Adv Biochem Eng Biotechnol.* 2007; 103:309–329. [PubMed: 17195468]
- Noshiro O, Hirayama R, Shimaya A, Yoneta T, Niigata K, Shikama H. Role of plasma insulin concentration in regulating glucose and lipid metabolism in lean and obese Zucker rats. *Int J Obes Relat Metab Disord.* 1997; 21(2):115–121. [PubMed: 9043965]
- Petrescu I, Bojan O, Saied M, Barzu O, Schmidt F, Kuhnle HF. Determination of phosphoenolpyruvate carboxykinase activity with deoxyguanosine 5'-diphosphate as nucleotide substrate. *Anal Biochem.* 1979; 96(2):279–281. [PubMed: 474956]
- Qu B, Li QT, Wong KP, Tan TM, Halliwell B. Mechanism of clofibrate hepatotoxicity: Mitochondrial damage and oxidative stress in hepatocytes. *Free Radic Biol Med.* 2001; 31(5):659–669. [PubMed: 11522451]
- Qureshi K, Abrams GA. Metabolic liver disease of obesity and role of adipose tissue in the pathogenesis of nonalcoholic fatty liver disease. *World J Gastroenterol.* 2007; 13(26):3540–3553. [PubMed: 17659704]
- Raman P, Donkin SS, Spurlock ME. Regulation of hepatic glucose metabolism by leptin in pig and rat primary hepatocyte cultures. *Am J Physiol Regul Integr Comp Physiol.* 2004; 286(1):R206–R216. [PubMed: 14512272]
- Ross BD, Hems R, Krebs HA. The rate of gluconeogenesis from various precursors in the perfused rat liver. *Biochem J.* 1967; 102(3):942–951. [PubMed: 16742514]
- Salhanick AI, Chang CL, Amatruda JM. Hormone and substrate regulation of glycogen accumulation in primary cultures of rat hepatocytes. *Biochem J.* 1989; 261(3):985–992. [PubMed: 2679547]
- Shen C, Zhang G, Meng Q. Enhancement of the predicted drug hepatotoxicity in gel entrapped hepatocytes within polysulfone-g-poly (ethylene glycol) modified hollow fiber. *Toxicol Appl Pharmacol.* 2010; 249(2):140–147. [PubMed: 20816885]
- Shen C, Zhang G, Qiu H, Meng Q. Acetaminophen-induced hepatotoxicity of gel entrapped rat hepatocytes in hollow fibers. *Chem Biol Interact.* 2006; 162(1):53–61. [PubMed: 16797510]
- Soboll S. Regulation of energy metabolism in liver. *J Bioenerg Biomembr.* 1995; 27(6):571–582. [PubMed: 8746844]

- Tolman KG, Freston JW, Kupfer S, Perez A. Liver safety in patients with type 2 diabetes treated with pioglitazone: Results from a 3-year, randomized, comparator-controlled study in the US. *Drug Saf.* 2009; 32(9):787–800. [PubMed: 19670918]
- Toritsuka N, Nonogaki Y, Hori Y, Sawada S, Shoun H, Motooka S. Well maintained expression of CYP genes in sandwich-culturing hepatocytes: Quantitative analysis using real-time PCR method. *Res Commun Mol Pathol Pharmacol.* 2001; 110(5–6):311–318. [PubMed: 12889523]
- Tuschl G, Hrach J, Walter Y, Hewitt PG, Mueller SO. Serum-free collagen sandwich cultures of adult rat hepatocytes maintain liver-like properties long term: A valuable model for in vitro toxicity and drug-drug interaction studies. *Chem Biol Interact.* 2009; 181(1):124–137. [PubMed: 19482013]
- Walker PR, Grindle MJ. Effects of hormones and serum on glycogen metabolism in adult rat liver parenchymal cell primary cultures. *J Cell Physiol.* 1977; 91(2):181–191. [PubMed: 405398]
- Wang HZ, Chang CH, Lin CP, Tsai MC. Using MTT viability assay to test the cytotoxicity of antibiotics and steroid to cultured porcine corneal endothelial cells. *J Ocul Pharmacol Ther.* 1996; 12(1):35–43. [PubMed: 8925394]
- Wen YA, Liu D, Xiao YY, Luo D, Dong YF, Zhang LP. Enhanced glucose synthesis in three-dimensional hepatocyte collagen matrix. *Toxicol In Vitro.* 2009; 23(4):744–747. [PubMed: 19268699]
- Wilkening S, Bader A. Influence of culture time on the expression of drug-metabolizing enzymes in primary human hepatocytes and hepatoma cell line HepG2. *J Biochem Mol Toxicol.* 2003; 17(4):207–213. [PubMed: 12898644]
- Wu DQ, Zhang GL, Shen C, Zhao Q, Li H, Meng Q. Evaluation of diffusion in gel entrapment cell culture within hollow fibers. *World J Gastroenterol.* 2005; 11(11):1599–1604. [PubMed: 15786534]
- Wu FJ, Friend JR, Remmel RP, Cerra FB, Hu WS. Enhanced cytochrome P450 IA1 activity of self-assembled rat hepatocyte spheroids. *Cell Transplant.* 1999; 8(3):233–246. [PubMed: 10442736]
- Yamada S, Otto PS, Kennedy DL, Whayne TF Jr. The effects of dexamethasone on metabolic activity of hepatocytes in primary monolayer culture. *In Vitro.* 1980; 16(7):559–570. [PubMed: 6105997]
- Yang Y, Xiao M, Mao Y, Li H, Zhao S, Gu Y, Wang R, Yu J, Zhang X, Irwin DM, Niu G, Tan H. Resistin and insulin resistance in hepatocytes: Resistin disturbs glycogen metabolism at the protein level. *Biomed Pharmacother.* 2009; 63(5):366–374. [PubMed: 18672341]
- Yin HQ, Kim M, Kim JH, Kong G, Lee MO, Kang KS, Yoon BI, Kim HL, Lee BH. Hepatic gene expression profiling and lipid homeostasis in mice exposed to steatogenic drug, tetracycline. *Toxicol Sci.* 2006; 94(1):206–216. [PubMed: 16917069]
- Yki-Jarvinen H. Thiazolidinediones and the liver in humans. *Curr Opin Lipidol.* 2009; 20(6):477–483. [PubMed: 19779336]

Figure 1.

Morphology examination of rat hepatocyte organoids. Bright field images at different culture time (A–C), and SEM (D and E)/TEM (F) images organoids at 24 h of culture. Hepatocytes organoid were wrapped by collagen fibers around the outer surface (white arrow) (E), the ultrastructures were indicated by different characters (F), nucleus (N), mitochondria (M), microvillus (m), and bile-canaliculus (bc).

Figure 2.

Glucose consumption (A) and glycogen content (B) in each culture model. Data are given as mean $\pm$ SD. Each data has three replicates with cells from three different rats. The asterisk (*) indicates a statistically significant difference between the monolayer culture and the sandwich/organoid culture (* P < 0.05; ** P < 0.01). # indicates a statistically significant difference between the sandwich culture and the organoid culture (# P < 0.05; ## P < 0.01).

Figure 3.

PEPCK activity in each culture model at 2 and 4 days. Data are given as mean $\pm$ SD.

Asterisk (*) indicates a statistically significant difference between the monolayer culture and the sandwich/organoid culture (* P < 0.05; ** P < 0.01). # indicates a statistically significant difference between the sandwich culture and the organoid culture (# P < 0.05; ## P < 0.01).

Figure 4. RT-PCR analysis of GAPDH, G6Pase, Gck, Glut2, Gys2, and Pck1 mRNA were respectively expressed as a relative to in GAPDH in each culture model at 2 and 4 days. Data are given as mean $\pm$ SD. Note that results were confirmed with cells isolated from two different rats.

Figure 5.

Hepatic glycogen content of each culture model in response to different hormone treatment.

A: insulin treatment at 5 or 20 mM glucose; **B:** glucagon treatment at the presence of insulin and glucose; **C:** dexamethasone treatment at the presence of insulin and glucose. The corresponded concentration of insulin, glucagon and dexamethasone were 10, 1, and 100 nM, respectively. Data are given as mean $\pm$ SD. Asterisk (*) indicates a statistical difference from values without hormone addition in the same culture group, * $P < 0.05$, ** $P < 0.01$.

Figure 6.

Effect of glucose concentration on lipid accumulation (**A** and **B**), reactive oxidative stress (**C** and **D**), MTT (**E**) and MMP (**F**) under monolayer and organoid after 6-day culture. Data are given as mean $\pm$ SD. The asterisk (*) indicates a statistical difference from 5.5 mM value in the same culture group, * $P < 0.05$, ** $P < 0.01$.

Table 1
Genes and corresponding primer sequence used for reverse-transcriptional PCR analysis.

Target (accession no.)	Description	Function	Primer sequence (show from 5' to 3' end)	Amplicon size (bp)
GAPDH (AB017801.1)	Glyceraldehyde-3-phosphate dehydrogenase	Housekeeping gene	For: AAGGTCAATCCCAGAGCTGAA Rev: GGATGGAAATGTGAGGGAGA	475
G6pc (D78592)	Glucose-6-phosphatase	Gluconeogenesis/glycogenolysis	For: TCTTTCCCATCTGGTTCCAC Rev: AAAGTGAGCCGCCAAGGTAGA	395
Gck (J04218)	Glucokinase	Glycolysis/glycogen synthesis	For: GCAGAAAGGGAAACAAACATCGT Rev: TCTCCGTGGAAACAGAAGGTT	431
Glut2 (BC078875.1)	Glucose transporter 2	Glucose transporter	For: GTTTTGGGTGTTCTCTCTGGA Rev: TGATCCTTCCGAGTTTGTC	238
Gys2 (AF346902.1)	Glycogen synthase	Glycogenesis	For: TTTCTCTGGGAAGTGACCAAC Rev: TTTGCTGCACAGAGATACCG	553
Pck1 (BC081900.1)	Phosphoenolpyruvate carboxykinase	Gluconeogenesis	For: ATGCTGATCCTGGGCATAAC Rev: TCGATGCCTTCCCAGTAAAC	329

Shown are sequences of forward (for.) and reverse (rev.) primer for each target.

Table II

Liver-specific functions of rat hepatocytes in each culture model.

Culture days	Albumin secretion (mg/g protein)				Urea synthesis (mg/g protein)			
	2 days	4 days	8 days	14 days	2 days	4 days	8 days	14 days
Monolayer	0.23 ± 0.10	0.35 ± 0.09	0.37 ± 0.26	0.34 ± 0.35	4.79 ± 0.42	9.58 ± 1.80	16.35 ± 1.22	18.11 ± 2.25
Sandwich	0.25 ± 0.02	0.63 ± 0.19*	1.02 ± 0.35**	2.15 ± 0.56**	8.57 ± 1.14**	14.5 ± 2.04*	21.46 ± 1.82*	26.25 ± 2.11*
Organoid	0.64 ± 0.11***##	1.23 ± 0.06***##	2.44 ± 0.61***##	3.51 ± 0.45***, #	9.58 ± 0.60***, #	27.54 ± 2.40***##	40.62 ± 3.32***##	50.34 ± 2.03***##

Asterisk (*) indicates a statistically significant difference between the monolayer culture and the sandwich/organoid culture (* P < 0.05; ** P < 0.01);

# indicates a statistically significant difference between the sandwich culture and the organoid culture (# P < 0.05; ## P < 0.01).

Table III

Glycogenolysis and gluconeogenesis in each culture model after 2 days of incubation.

Cellular function	Culture model			In vivo
	Monolayer	Sandwich	Organoid	
Glycogenolysis rate (μmols glucose/min/g protein)				
Without glucagon	0.45 ± 0.05	0.72 ± 0.30*	1.25 ± 0.08**,#	~2.5 (Ikezawa et al., 1998)
With glucagon	0.55 ± 0.08	0.81 ± 0.15*	1.95 ± 0.09**.,##	~5 (Ikezawa et al., 1998)
Gluconeogenesis rate (μmols glucose/min/g protein)				
With glycerol	0.70 ± 0.07	1.01 ± 0.11	3.20 ± 0.12**.,##	2.4 (Ross et al., 1967)
With pyruvate	1.80 ± 0.03	1.43 ± 0.13	4.45 ± 0.11**.,##	5.1 (Ross et al., 1967)

Data are given as mean $\pm$ SD. Asterisk (*) indicates a statistically significant difference between the monolayer culture and the sandwich/organoid culture (* P < 0.05; ** P < 0.01).

# indicates a statistically significant difference between the sandwich culture and the organoid culture ($^{\#}P$ < 0.05; $^{\#\#}P$ < 0.01).

Table IV

Glucose metabolism related functions of hepatocytes in each culture model compared to in vivo.

Cellular function	<u>Similarity with the in vivo behavior</u>		
	Monolayer	Sandwich	Organoid
Glucose metabolism properties			
Glucose consumption	+	++	++
Glycogen content	+	+	++
Gluconeogenesis	+	+	+++
Hormonal response			
Insulin	+	+	++
Glucagon	–	–	++
Dexamethasone	–	–	++
Hyperglycemia response			
Oxidative stress	–	n.d.	++
Lipid accumulation	–	n.d.	++
Mitochondrial damage	–	n.d.	+

The number of “+” means the similarity with the in vivo condition, the more the closer, while “–” means the in vivo response could not observed in vitro; “n.d.” stands for not detected.