Lessons learnt in establishing a reliable and low-cost assay for urea production in human primary hepatocytes cultured in a Liver-Chip for the study of drug hepatotoxicity

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Abstract

Background: Liver-on-a-chip (Liver-Chip) is a microphysiological system (MPS) designed to better maintain hepatic functions for liver cells cultured under in vitro conditions. Urea is synthesized exclusively by hepatocytes and is a proposed quality control marker for hepatocyte function maintained in MPS. Purpose: This study aimed to investigate the sensitivity and specificity of two urea assay methods for cells cultured in a Liver-Chip system for the study of acetaminophen-induced liver injury. Methodology: Primary human hepatocytes (PHHs) were co-cultured with three types of non-parenchymal cells (NPCs) including liver sinusoid endothelial cells, Kupffer and stellate cells in Emulate S1-Chips®. The perfusing culture medium, also called effluent, was collected every 24 h to measure urea levels. Chemical and enzymatic methods, which have been reported for measuring urea in S1-Chips, were tested in parallel in the same samples. Results: In the enzymatic method, culture medium alone led to a 6-fold increase of signal in urea measurements compared to blank buffer controls. Such background signals were directly proportional to levels of sodium pyruvate, a common component of many types of culture medium, including PHH cell cultures. This high background signal introduced large variations among replicates of the same sample, making it impossible to accurately determine the relatively low levels of urea, which were about 10 µg/mL, in the S1-Chips effluents. Effluents must be diluted by 5-fold to effectively reduce such interference, but this made it difficult to observe the percentage of reduction after drug treatment, as urea levels neared the limit of detection (1.2 µg/mL). In contrast, the chemical method had negligible background signal for culture medium and a lower limit of detection (0.8 µg/mL). Additionally, the urea level could be accurately determined by chemical method at a fraction of the cost of the enzymatic method. As determined by the chemical method, the average urea production rate of hepatocytes from three human donors was 203 (± 29) µg/million cells/day, and acetaminophen treatment (0.1 to 10 mM) led to a concentration- and time-dependent reduction of 20% to 60%. Conclusion: These data may guide selection of appropriate assays and aid optimization of procedures for urea detection in various liver cell culture platforms.

Introduction

Liver-on-a-chip (Liver-Chip) is a microphysiological system (MPS) designed to culture hepatic cells in a three-dimensional environment on microfluidic channels, enabling better maintenance of primary liver cells and thus improved prediction of drug-induced liver injury. Urea is exclusively synthesized by hepatocytes and is therefore a proposed endpoint reflecting the performance of Liver-Chip platforms in maintaining hepatic functions. Numerous assay kits are available for measuring urea levels in in vitro studies of DILI, but the reliability and reproducibility of the results for cells maintained in MPS need to be evaluated. In our study of acetaminophen hepatotoxicity, we have been using an Emulate® Liver-Chip, which cocultures primary human hepatocytes (PHHs) with liver non-parenchymal cells (NPCs) consisting of sinusoidal endothelium, Kupffer, and Stellate cells. This project aims to (1) establish a reproducible and sensitive assay to measure the urea level in the perfusing medium of primary human hepatocytes (PHHs) cultured in Emulate® Liver-Chips (2) observe the effect of acetaminophen (APAP) on urea production in PHHs maintained in the Liver-Chip.

Materials and Methods

- PHHs and primary human liver non-parenchymal cells (NPCs) including liver sinusoidal endothelium (LSEC), Kupffer, and stellate cells were cocultured in the Emulate S1-Chips®.
- Cells were treated with APAP at a range of concentrations for 7 consecutive days. The vehicle control was cell culture medium. The APAP concentrations included:
    i. 0.1 mM - the human therapeutical peak blood level
    ii. 1.0 mM - the human blood concentration that causes liver injury and needs clinical intervention
    iii. 3.0 mM - the highest reported blood concentrations in humans who suffered acute liver injury due to APAP overdose
    iv. 10.0 mM - the toxic concentration that causes massive cell death to PHHs in vitro.
- The perfusing medium, with or without APAP, was refreshed every 48 h. The spent medium, also called effluent, was collected every 24 h after the first treatment for urea detection until experiments were terminated.
- Two commercially available assay kits were used to measure urea levels in the effluent. The two kits represent two widely used approaches for urea detection. Specifically, a Sigma kit (Catalog number: MAK006) was used for the enzymatic approach and a kit from the BioChain Institute Inc. (Catalog number: Z5030016) was used for the chemical method.
- The same effluent was aliquoted for urea detection using different methods

Results and Discussion

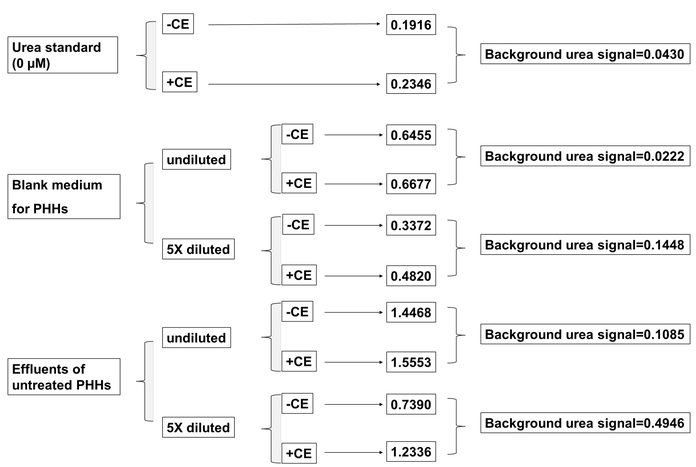

Figure 1. Background signals in the enzymatic assay for urea detection using the culture medium for PHHs maintained in Emulate® Liver-Chips. The Sigma kit (Catalog number: MAK006) was used to measure urea level in the PHH culture medium. The assay was performed by including different control groups following the manufacturer protocol. The blank medium and effluent were either undiluted or diluted by 5-fold using the buffer provided in the kit for the purpose of examining possible interfering effects from medium ingredients. CE, converting enzyme provided by the kit; –CE, without CE; +CE, with CE. The value presented are the means of two to four independent experiments (n=2 to 4).

Results and Discussion

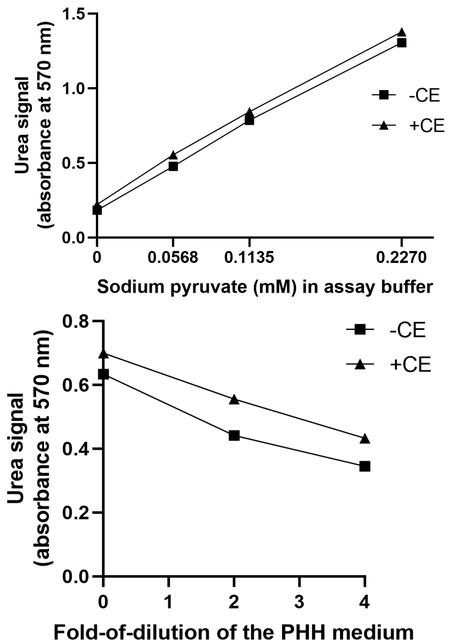

Figure 2. Sodium pyruvate was a possible source of the background signal in the enzymatic assay for urea detection using PHH medium. The Sigma kit (Catalog number: MAK006) was used to perform urea assay. In panel A, the assay buffer provided by the kit was supplemented with different concentrations of sodium pyruvate and used for urea detection. The highest concentration of sodium pyruvate supplemented was equal to those in the PHH medium, that is 0.2270 mM. In panel B, the PHH medium was diluted using the assay buffer from the kit and then used to measure urea signal. CE, converting enzyme provided by the kit; –CE, without CE; +CE, with CE. The value presented are means of two to four independent experiments (n=2 to 4).

Disclaimer

This presentation reflects the views of the authors and does not necessarily reflect those of the U.S. Food and Drug Administration. Any mention of commercial products is for clarification only and is not intended as approval, endorsement, or recommendation.

Acknowledgements

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	Undiluted	5X diluted
Sigma kit: ±CE	2.58	182.46
Sigma kit: +CE	104.69	435.94
BioChain kit	186.22	Below detection limit

Table 1. Comparison of urea levels detected using enzymatic and chemical method. The effluent from untreated PHH was evenly divided and used to measure urea levels using the Sigma and BioChain kit in parallel experiments. CE, converting enzyme provided by the kit; ±CE, urea level was calculated using the difference between signals obtained with and without CE; +CE urea level was calculated using the signal obtained with CE only. PHH effluent was collected two days after the perfusing culture started. Effluents from 24 Liver-Chips were pooled for the study. The unit of urea was µg/million cells/day. The detection limit of the BioChain kit was 0.8 µg/mL Data are representative of PHH from one donor.

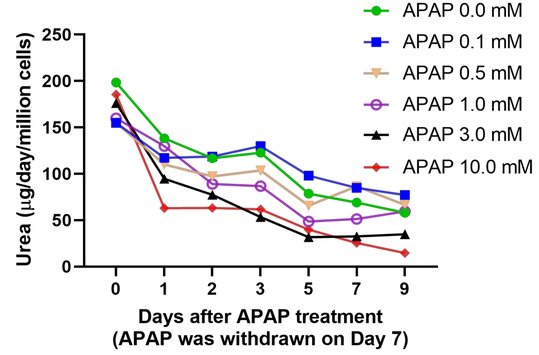

Figure 3. Effects of APAP on urea levels in the effluents of PHHs cultured in Emulate® Liver-Chips. PHHs and NPCs were cocultured in the top and bottom channels of Emulate® Liver-Chips, respectively. Cells were treated with APAP for 7 consecutive days and the effluent collected daily for urea measurement using the BioChain kit. Urea levels were normalized to the estimated number of PHHs in each Liver-Chip, that is, 42,000 PHHs per Chip. Urea was undetectable (below 0.8 µg/mL) in the effluents of NPCs cultured in the bottom channel (data not shown). Data are the means of 2-4 Chips in each treatment group.

Conclusion

1. The chemical method provided improved accuracy and enhanced sensitivity and was significantly less expensive than the enzymatic approach in detecting urea level in the spent culture medium of PHHs co-cultured with NPCs in the Emulate® Liver-Chips.
2. APAP caused time- and concentration-dependent decreases of urea production in PHHs maintained in the Emulate® Liver-Chips