A multi-organ-chip co-culture of human liver equivalents and neurospheres for long-term substance testing

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Abstract

Current *in vitro* and animal tests for drug development are failing to emulate the organ complexity of the human body and, therefore, to accurately predict drug toxicity. In this study, we present a smartphone-sized, self-contained multi-organ-chip (MOC) platform capable of co-cultivating up to three organ equivalents inside a combined media circuit. A peristaltic on-chip micro-pump reproducibly operates a PDMS-embedded microcirculation system, emulating the systemic arrangement of organs within the human body. It could be shown, that the multi-organ-chip is capable of supporting long-term co-cultures of human artificial liver microtissues and neurosphers. Cultures were successfully maintained functional over a period of up to 14 days. Liver cell polarity was restored as shown by the expression of specific transporters, tight junctions and the formation of rudimentary bile canalicular-like structures. Vitality of the cells was assessed by TUNEL/Ki 67 staining and was markedly increased compared to static controls. Neurospheres derived from the Ntera-2 (NT2) cell line were strongly positive for neuronal markers MAP2 and ß-Tubulin III after 14 days of culture in the MOC as assessed by immunohistology and qPCR. Chronic exposure of the cultures to 2,5-hexandione over 14 days revealed a dose-dependent toxicity on MOC co-cultures.

Experimental Set Up

Generation of tissue equivalents

Liver aggregates containing 4.8 x 10^4 HepaRG cells and 0.2 x 10^4 human hepatic stellate cells (HHSteC) were formed in Perfecta3D® 384-Well Hanging Drop Plates (3D Biomatrix, USA). After two days of hanging drop culture, 20 aggregates were loaded into a single tissue culture compartment of the micro-bioreactor.

Neurospheres were produced from undifferentiated NT2 cells. Therefore, 7x10^5 cells/ml were inoculated in a silanized 125 ml spinner vessel equipped with ball impeller. Differentiation was induced by 10 µM retinoic acid for 3 weeks. During the differentiation period, a 50% medium exchange was performed every 2-3 days.

Bioreactor culture

Each circuit of the micro-bioreactor device contained 700 µl medium in total. Daily samples were collected for respective analyses. Experiments were stopped at day 14 and tissues were subjected to immunohistochemical stainings. Experiments were conducted with four replicates.

Tissue exposure to 2,5-hexanedione

Co-cultures were exposed to 0 mM, 16 mM and 32 mM 2,5-hexandione, starting on day 6 of MOC culture. Substance application was repeated at 24 h intervals simultaneously with the medium change.

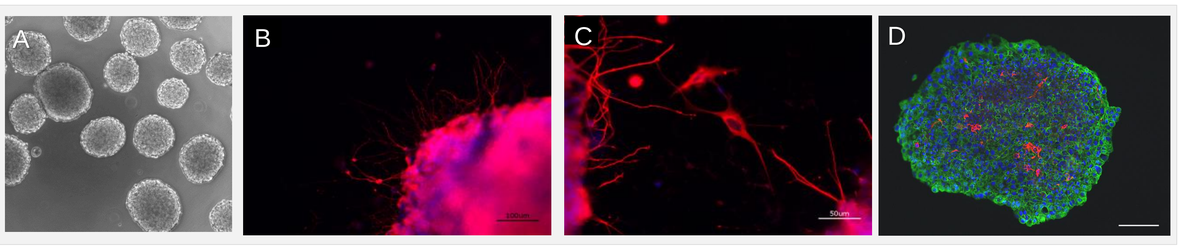

Fig. 1. Characterization of tissue equivalents. (A) Light microscopy of neurospheres. Immunofluorescent staining of outgrowing neurospheres for **(B)** MAP2 (red) and **(C)** ß-Tubulin III (red). Liver equivalents were stained for **(D)** cytokeratin 8/18 (green) and vimentin (red). Nuclei are stained with hoechst 33342. Scale bars B, D 100 µm, C 50 µm.

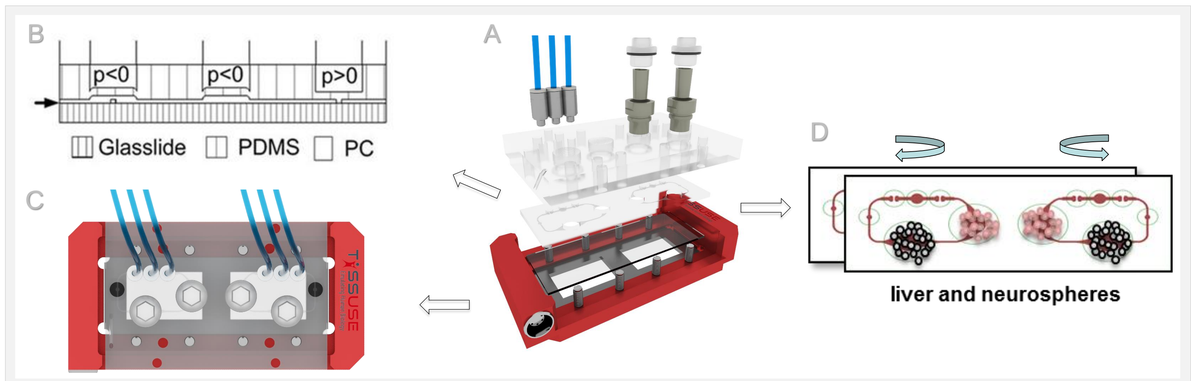

Fig. 2. The microfluidic MOC device at a glance. (A) Exploded view of the micro-bioreactor comprising a polycarbonate cover-plate, the PDMS-glass chip accommodating two microfluidic circuits and a heatable MOC-holder (red). **(B)** Cross-section of a peristaltic on-chip micropump (thickness of valves: 500 µm). **(C)** Model of the assembled microfluidic device. **(D)** Top view of the MOC layout illustrating the two separate microfluidic circuits (channel height: 100 µm; width: 500 µm) each accommodating two cell-culture insert areas. Arrows indicate the direction of flow.

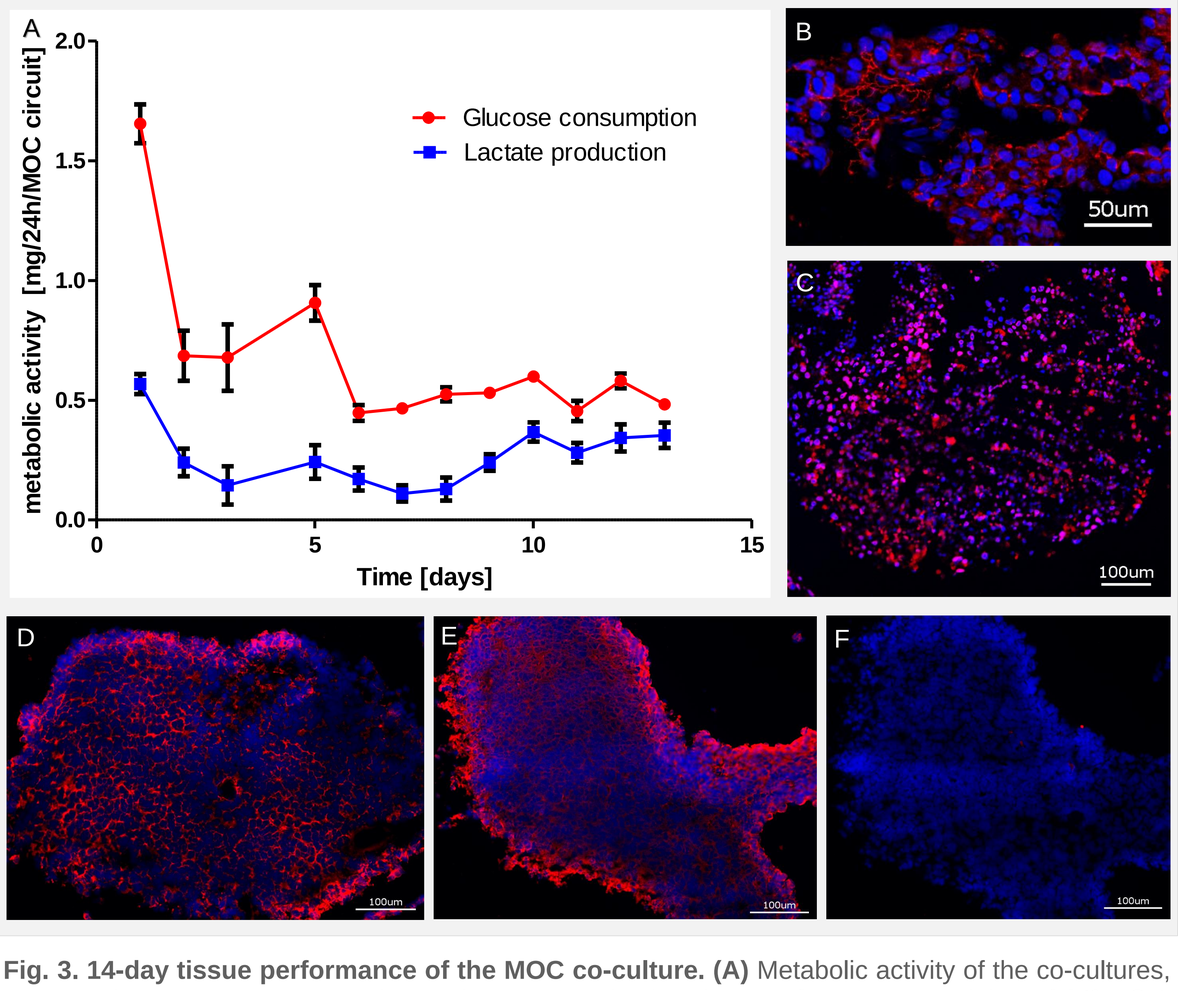

Fig. 3. 14-day tissue performance of the MOC co-culture. (A) Metabolic activity of the co-cultures, measured as glucose consumption and lactate production. Immunofluorescent staining of **(B)** ZO-1 (red) and **(C)** cytochrome P450 3A4 (red) in liver equivalents. Neurospheres were stained for **(D)** MAP2 (red), **(E)** ß-Tubulin III (red) and **(F)** TRA-1-60 (red). Nuclei are stained with hoechst 33342. Scale bars B 50 µm, C, D, E, F 100 µm.

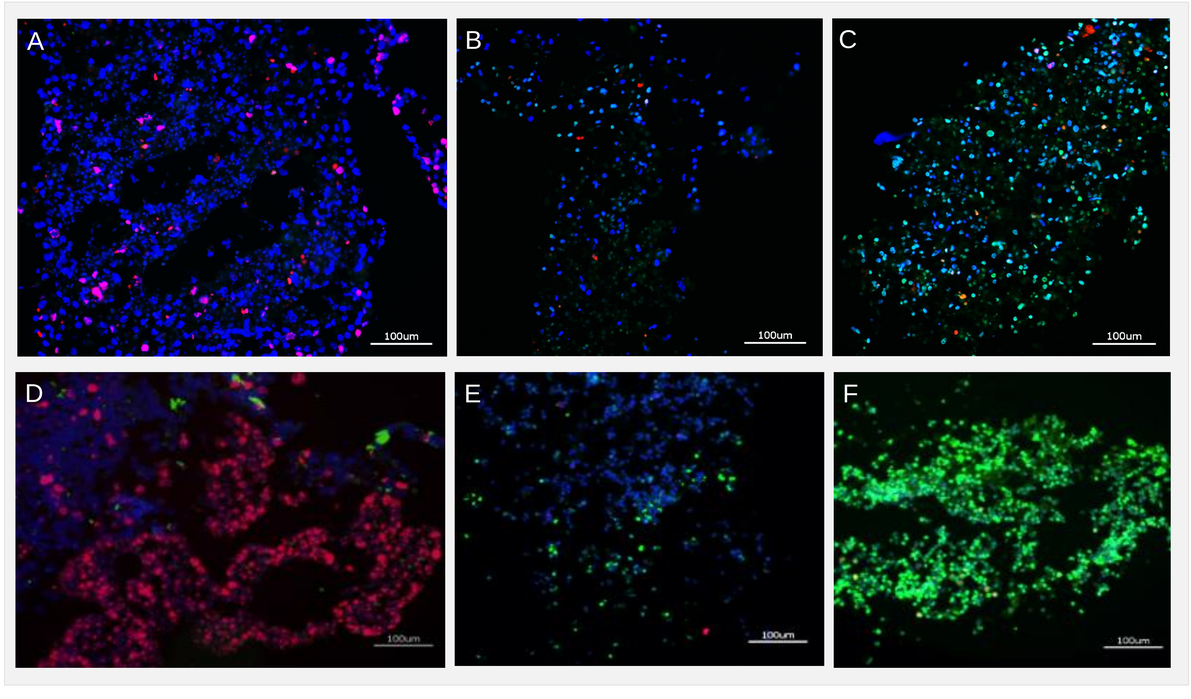

Fig. 4. 14-day MOC tissue sensitivity to 2,5-hexanedione. Sensitivity to 2,5-hexandione after 9 days of repeated dose exposure was shown by TUNEL / Ki67 staining. **(A, B, C)** liver equivalents and **(D, E, F)** neurospheres from MOC co-cultures treated with **(A, D)** 0, **(B, E)** 16 mM and **(C, F)** 32 mM substance. Nuclei are stained with hoechst 33342. Scale bars 100 µm.

Results

Co-cultures of human artificial liver microtissues, consisting of HepaRG cells and HHSteC, and neurospheres have successfully been cultivated over 14 days in the novel microfluidic bioreactor. Glucose consumption and lactate production indicated an aerobic metabolism which reached a steady state after 6 days. Immunohistochemical staining revealed the expression of phase I metabolic enzyme cytochrome P450 3A4 throughout the whole liver equivalent and a network-like arrangement of tight junction protein ZO-1 indicating the polarization of hepatocytes. Neurospheres stained strongly positive for neuronal markers MAP2 and ß-Tubulin III, whereas the embryonic stem cell marker TRA-1-60 was negative in all samples. The intensity of staining was comparable to control neurospheres at day zero, which were not cultivated in the MOC. Furthermore, the cultures revealed a dose-dependent response to a 9-day exposure to the toxic substance 2,5-hexanedione as shown by TUNEL / KI67 staining.

Summary

A promising tool for long term co-culture of human liver equivalents and neurospheres has been developed. The simple MOC design presented, operated cultures at a total on-chip volume of 700 µl medium at recirculation rates of 40 µl/min assisted by an on-chip micropump.

The prediction of toxicology profiles of compounds was demonstrated possible by exposing the cells to different concentrations of 2,5-hexanedione.

This platform is designed to generate high-quality *in vitro* data predictive of substance safety in humans. Tissue cultures can be exposed to pharmaceutical substances at regimens relevant to respective guidelines, currently used for subsystemic substance testing in animals.

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