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

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ABSTRACT

Current *in vitro* and animal tests for drug development are failing to emulate the systemic organ complexity of the human body and, therefore, often do not accurately predict drug toxicity, leading to high attrition rates in clinical studies (Paul et al., 2010). The phylogenetic distance between humans and laboratory animals is enormous, this affects the transferability of animal data on the efficacy of neuroprotective drugs. Therefore, many neuroprotective treatments that have shown promise in animals have not been successful when transferred to humans (Dragunow, 2008; Gibbons and Dragunow, 2010). We present a multi-organ chip capable of maintaining 3D tissues derived from various cell sources in a combined media circuit which bridges the gap in systemic and human tests. A steady state co-culture of human artificial liver microtissues and human neurospheres exposed to fluid flow over two weeks in the multi-organ chip has successfully proven its long-term performance. Daily lactate dehydrogenase activity measurements of the medium and immunofluorescence end-point staining proved the viability of the tissues and the maintenance of differentiated cellular phenotypes. Moreover, the lactate production and glucose consumption values of the tissues cultured indicated that a stable steady-state was achieved after 6 days of co-cultivation. The neurospheres remained differentiated neurons over the two-week cultivation in the multi-organ chip, proven by qPCR and immunofluorescence of the neuronal markers βIII-tubulin and microtubule-associated protein-2. Additionally, a two-week toxicity assay with a repeated substance exposure to the neurotoxic 2,5-hexanedione in two different concentrations induced high apoptosis within the neurospheres and liver microtissues, as shown by a strong increase of lactate dehydrogenase activity in the medium. The principal finding of the exposure of the co-culture to 2,5-hexanedione was that not only toxicity profiles of two different doses could be discriminated, but also that the co-cultures were more sensitive to the substance compared to respective single-tissue cultures in the multi-organ-chip. Thus, we provide here a new *in vitro* tool which might be utilized to predict the safety and efficacy of substances in clinical studies more accurately in the future.

1. Introduction

The development of new drugs is strikingly expensive, time-consuming and only a few substances can stand up to the long testing phase. Animal tests often fail to forecast the effects of new drugs, because the phylogenetic distance between laboratory animals and humans is too great (Bailey et al., 2013; Dragunow, 2008). The large quantities of laboratory animals used for substance testing are not only expensive and laborious, but are also ethically questionable. Therefore, it is essential to find new technologies to

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evaluate potential drug candidates at an early stage of the process. Microfluidic culture devices combining human microtissues in an organ-like arrangement at a homeostatic steady-state could well become a translational solution for that testing dilemma.

Currently, there are very few microfluidic platforms available which mimic the human situation *in vivo* (Hwan et al., 2009; Zhang et al., 2009). The disadvantages of most of these systems are the external pump and external media container, leading to a high fluid-to-tissue ratio. Thus, the crosstalk between tissues is not ensured. Furthermore, the exposure time of substances or drugs to most of these microfluidic systems ranges between 24 and 72 h, and only a few can operate up for to seven days (Marx et al., 2012). However, the Organization for Economic Co-operation and Development guidelines for toxicity testing of chemicals and cosmetics in animals require a 28-day repeated dose exposure.

Here we evaluated whether the microfluidic multi-organ chip (MOC) platform developed in our laboratory is able to co-cultivate neurospheres and liver spheroids over prolonged culture periods. The MOC, covering the area of an object slide, contains an on-chip micropump and is capable of interconnecting two different organ equivalents. The micropump ensures a stable long-term circulation of media through the tissue culture compartments at variable flow rates, adjustable to mimic blood flow-dependent mechanical shear stress in the respective tissues (Schimek et al., 2013). The tissue culture compartments and the connecting channels are optically accessible, thus, supporting live tissue imaging. We show here that the MOC is able to support co-cultures of human liver spheroids, consisting of differentiated HepaRG cells and human hepatic stellate cells, and differentiated neurospheres, derived from the NT2 cell line, over weeks at a steady-state in a combined media circuit. The differentiated phenotype of the cells was preserved, as shown by immunofluorescence staining and qRT-PCR of selected marker genes. Furthermore, the system layout and chip design support repeated substance exposure for safety or efficacy test assay development.

In a two-week toxicity assay 2,5-hexanedione was used to study it's toxic effect on the tissues. In the human liver n-hexane is either metabolized to 1-hexanol or 3-hexanol in a detoxification pathway, or in a bio-activation pathway to 2-hexanol. The 2-hexanol is further metabolized in many oxidation steps into 2,5-hexanedione and other metabolites (Yin et al., 2013). Those are distributed into the bloodstream and reach other organs as kidney and brain. 2,5-hexanedione is the main component leading to neurotoxicity, because it has the longest retention period in the human body in comparison to the other metabolites. 2,5-hexanedione can structurally change neurofilaments by pyrrole adduct formation and resulting covalent cross-linking (Heijink et al., 2000). The neurotoxic effect has been shown in different studies on laboratory animals and cell lines (DeCaprio et al., 1988; Ladefoged et al., 1994). However, a four hour exposure of 2,5-hexanedione on neuronal (NT-2-N, SK-N-H) and non-neuronal cells (NT-2) did not show any cytotoxicity, but a 24 h exposure of 32 mM 2,5-hexanedione led to toxic effects on both cell types (Woehrling et al., 2006).

2. Materials and methods

2.1. Cell sources and maintenance

Cell culture components were purchased from PAA Laboratories (GE Healthcare Europe GmbH, Vienna, Austria) and cultures were incubated in HepaRG medium at 37 °C and 5% CO_2, unless otherwise stated. HepaRG cells were obtained from Biopredic International (Rennes, France) and maintained as described by Gripon et al. (2002). Briefly, cells were cultured in HepaRG medium, consisting of William's Medium E supplemented with 10% (v/v) fetal bovine serum (FBS), 100 units ml^{-1} penicillin, 100 $\mu g\,ml^{-1}$ streptomycin, 5 $\mu g\,ml^{-1}$ human insulin, 2 mM L-glutamine, and 5×10^{-5} M hydrocortisone hemisuccinate (Sigma-Aldrich, St. Louis, MO, USA). Undifferentiated cells were maintained in 75 cm^2 tissue culture flasks (Greiner Bio One, Germany) at a seeding density of 2×10^4 cells cm^{-2} for two weeks. Induction of differentiation was initiated by allowing the cells to reach confluence by maintaining the cells in growth medium for two weeks. Differentiation medium containing 2% (v/v) dimethyl sulfoxide (DMSO; Carl Roth GmbH, Karlsruhe, Germany) was then added for another two weeks. Human hepatic stellate cells (HHSteC) and their culture supplements were purchased from ScienCell Research Laboratories (Carlsbad, CA, USA). The cells were seeded at 5×10^3 cells cm^{-2} in poly-L-lysine-coated 75 cm^2 tissue culture flasks in stellate cell medium for maintenance, according to the manufacturer's instructions. Medium was exchanged every two to three days. Cells were harvested for further use at 90% confluence. NTera-2/cl.D1 (NT2) cells were obtained from American Type Cell Culture Collection (ATCC) and maintained as described by Brito et al. (2007). Briefly, undifferentiated cells were routinely cultured at a seeding density of 4–4.5 $\times 10^4$ cell cm^{-2} in DMEM, supplemented with 10% (v/v) FBS and 1% (v/v) penicillin-streptomycin (P/S; all purchased from Invitrogen).

2.2. Preparation of neurospheres and liver microtissues

Preparation of neurospheres was performed as described in Serra et al. (2009). Briefly, a single cell suspension of undifferentiated NT2 cells was cultivated at 7×10^5 cell ml^{-1} in 75 ml DMEM, 10% FBS, 1% P/S, in a silanized 125 ml spinner vessel (from Wheaton) equipped with ball impeller. On day two, 50 ml of fresh medium was added. After three days, differentiation was induced by incubation with 10 μM retinoic acid (RA), for three weeks. During the differentiation period, a 50% medium exchange was performed every two to three days, with DMEM containing 10% FBS, 1% P/S and 20 μM RA.

Preparation of liver microtissues was performed as described in Wagner et al. (2013). In brief, 20 μl cell suspension containing 4.8×10^4 HepaRG cells and 0.2×10^4 primary human hepatic stellate cells (HHSteC) were added to each access hole of a Perfecta3D® 384-Well Hanging Drop Plate (3D Biomatrix, Ann Arbor, MI, USA). After two days of hanging drop culture, the spheroids were transferred to ultra-low attachment 24-well plates (Corning, Lowell, MA, USA) with a maximum of 20 spheroids per well.

2.3. Multi-organ chip manufacturing and culture

Fabrication of the MOC was performed as described in Wagner et al. (2013). In brief, applying replica moulding of polydimethylsiloxane (PDMS) resulted in a single 2 mm high PDMS layer containing the respective microfluidic channel system, which was permanently bonded to a glass microscope slide (Menzel, Braunschweig, Germany) using low pressure plasma oxidation (Femto; Diener, Ebhausen, Germany). The PDMS layer consisted of two compartments for cultivating cells and three pump membranes (thickness: 500 μm). A pumping frequency of 1.5 Hz and a pressure of 0.6 bar was applied.

Liver microtissues (20 spheroids per insert) and neurospheres (80 spheroids per insert), each in 350 μl HepaRG medium, were loaded into separate culture compartments of each MOC circuit for co-culture. Additional MOCs were loaded only with liver microtissues or neurospheres for comparison (Fig. 1). During the first five days, a 40% media exchange rate was applied at 12 h intervals. Later, a 40% exchange rate was applied every 24 h. Daily media samples were collected for lactate dehydrogenase (LDH), glucose and lactate