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Assessment Of Troglitazone Induced Liver Toxicity In A Dynamically Perfused Two-Organ Micro-Bioreactor System

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Abstract
The ever-growing amount of new substances released to the market and the limited predictability of current *in vitro* test systems has led to an ample need for new substance testing solutions. Many drugs like troglitazone, that had to be removed from the market due to drug induced liver injury, show their toxic potential only after chronic long term exposure. But for long-term multiple dosing experiments, a controlled microenvironment is pivotal, as even minor alterations in extracellular conditions may greatly influence the cell physiology.
Within our research program, we focused on the generation of a micro-engineered bioreactor, which can be dynamically perfused by an on-chip pump and combines at least two culture spaces for multi-organ applications. This circulatory systems better mimics the in vivo conditions of primary cell cultures and assures steadier, more quantifiable extracellular signaling to the cells.

Experimental Set Up

Transwell® multi-tissue culture in the MOC device over 28 days
Liver microtissues (aggregates of HepaRG+human hepatic stellate cells) and skin biopsies were cultured in separate inserts of a 96-well Transwell® unit (Corning), which were hung inside the chip with the membrane fitting directly over the circuit (fig. 1D). The tissue was cultivated either air/liquid interfaced (skin) or submerged in media (liver equivalent).

Multi-tissue exposure to troglitazone
Liver microtissue and skin biopsy co-cultures in MOCs were prepared as shown in fig. 1E. The tissues were cultured for one day in normal medium and were, subsequently, exposed to 0 µM, 5 µM and 50 µM troglitazone, respectively.

Application of troglitazone was repeated at 12 h intervals simultaneously with the medium change.

Co-culture of liver, skin and endothelial cells: microvascular circuits in the MOC
HDMECs, isolated from human foreskin, were seeded into the microfluidic channel system using a syringe. After even cell infusion inside the circuit the device was incubated in 5% CO_2 at 37°C under static conditions for 3 h to allow the cells to attach to the channel walls. A frequency of 0.476 Hz was applied for continuous dynamic operation, after 10 days of monoculture, skin and liver tissue were added for co-cultivation for another 15 days.

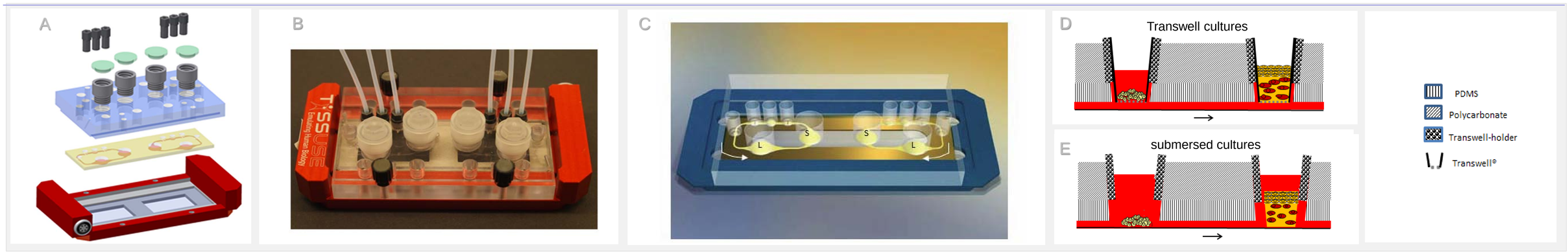

Fig. 1. The microfluidic MOC device at a glance. (A) Exploded view of the device comprising a polycarbonate cover-plate (blue), the PDMS-glass chip accommodating two microfluidic circuits (yellow; footprint: 76 mm x 25 mm; height: 3 mm) and a heatable MOC-holder (red). **(B)** Photograph of the assembled microfluidic device. **(C)** 3D drawing of an assembled MOC. Arrows indicate fluid flow direction of each circuit. S – skin culture compartments, L –liver culture compartments **(D and E)** Schematic sections through the tissue culture compartments supporting cultures in Transwells® (D) or submersed cultures (E) in the fluid flow.

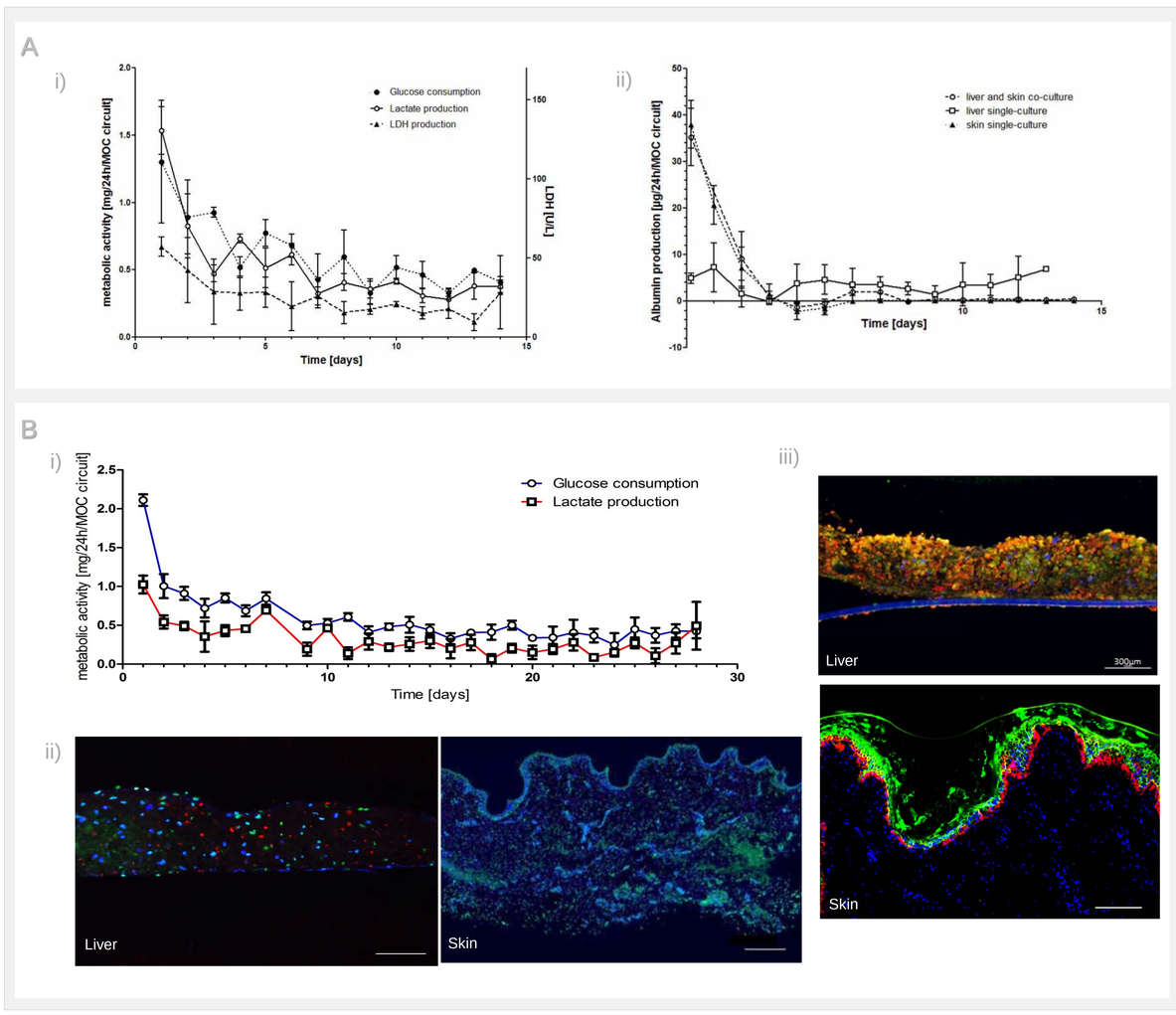

Fig. 2. Multi-tissue culture in the MOC device. (A) 14-day tissue performance at direct exposure to fluid flow. a) Glucose, lactate and LDH metabolic activity of MOC co-cultures. b) Albumin profiles of liver and skin single-cultures and liver and skin co-cultures in MOC. Data are means ± SEM (n = 6) **(B)** Liver and skin tissue performance over 28-day MOC co-culture. i) Metabolic activity of the co-culture analysed in media supernatants. ii) Cell viability shown by TUNEL/KI67 staining of liver tissue and TUNEL staining of skin tissue after 28-day co-culture. iii) Cell functionality shown by immunostaining of Phase I enzymes CYP450 3A4 (red) and CYP450 7A1 (green) in liver tissue and CK15 (red) and CK10 (green) showing undifferentiated and differentiated epidermis.

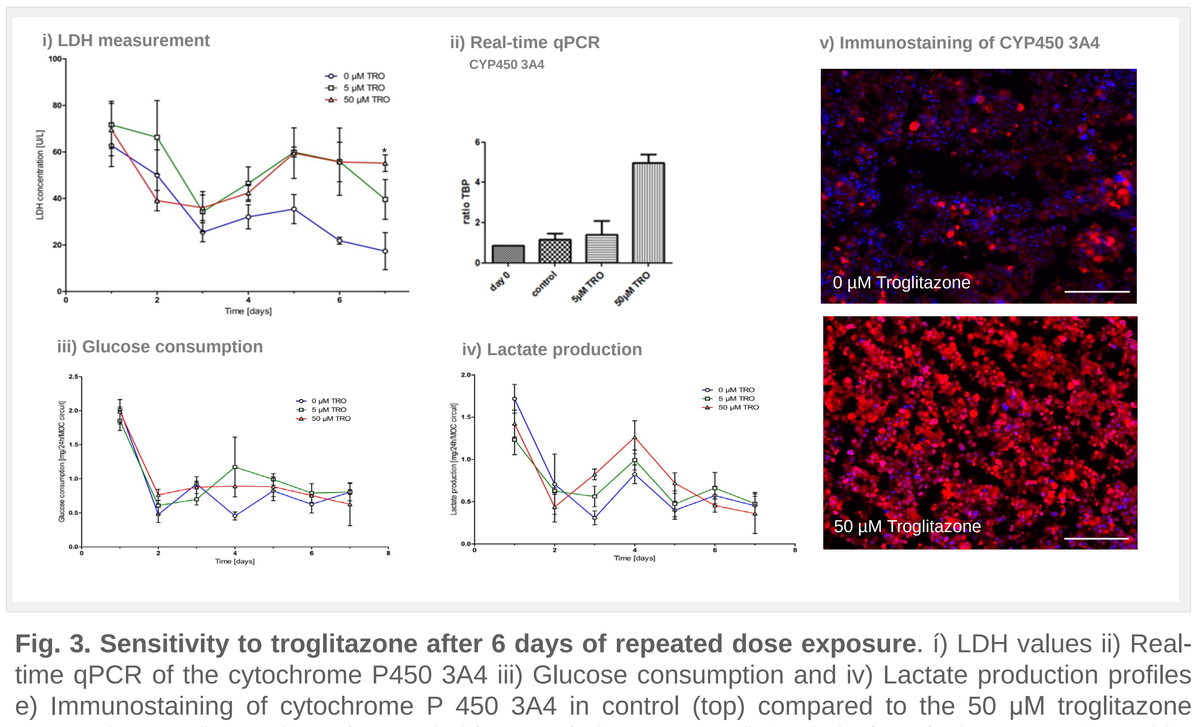

Fig. 3. Sensitivity to troglitazone after 6 days of repeated dose exposure. í) LDH values ii) Real-time qPCR of the cytochrome P450 3A4 iii) Glucose consumption and iv) Lactate production profiles e) Immunostaining of cytochrome P 450 3A4 in control (top) compared to the 50 µM troglitazone exposed group (bottom), nuclear stain blue. Scale bar 100 µm. i) Statistical analysis was performed by one-way analysis of variance (ANOVA), followed by *post-hoc Dunnett's pairwise multiple comparison test. * P<0.05 versus control. (a, b and c) Data are means ± SEM (n = 4).*

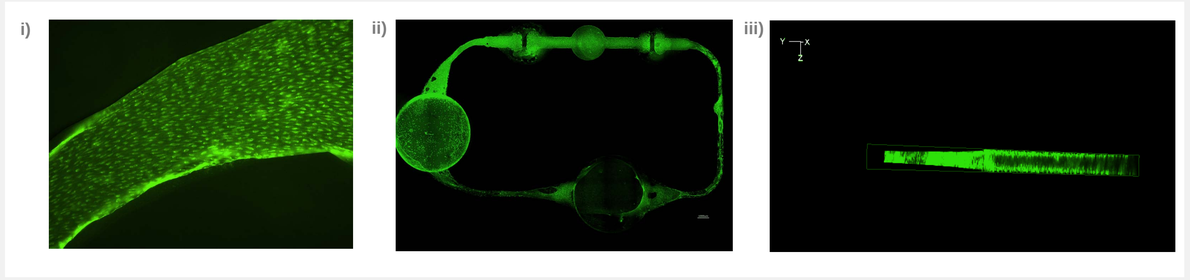

Fig. 4. Co-culture of liver, skin and endothelial cells: microvascular circuits in the MOC. (i) Cell adherence (HDMECs) to the bottom of the channels after 15 days of coculture. Bundles of stress fibres align along the direction of flow **(ii)** Functionality of the microvascular vessel system demonstrated by live cell viability staining (Calcein AM assay) endothelial cells maintain a full monolayer for 15 d of dynamic culture **(iii)** Image stack showing HDMECs covering the complete channel system. Scale bar = 1000 µm.

Results
Co-cultures of human artificial liver microtissues and skin biopsies have successfully proven the long-term performance of the novel microfluidic multi-organ-chip device. Furthermore, the co-cultures revealed a dose-dependent response to a 6-day exposure to the toxic substance troglitazone. Liver microtissues showed sensitivity at different molecular levels.
In addition, a robust procedure applying pulsatile shear stress has been established to cover all fluid contact surfaces of the system with a functional, tightly closed layer of HDMECs and co-cultivation of liver, skin and endothelial cells for 15 days was sucessful.

Summary
A unique chip-based tissue culture platform has been developed enabling the testing of drugs or chemicals on a set of miniaturized human organs. This “human-on-a-chip” platform is designed to generate high-quality in vitro data predictive of substance safety in humans. Tissue co-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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