Temporal Characterization of a 3D Bioprinted Model May Provide New Insight into Events Underlying Fibrotic Liver Injury

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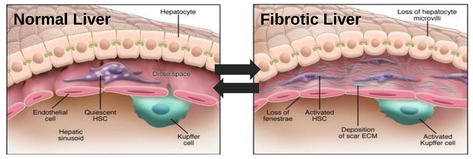
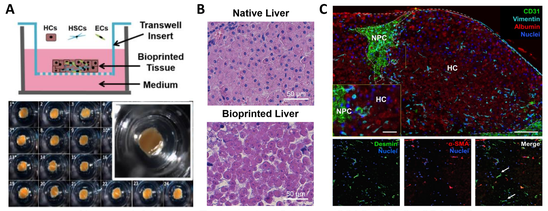
Introduction

- Hepatic fibrosis is an abnormal wound healing response orchestrated by complex intercellular interactions among hepatocytes (HCs), endothelial cells (ECs), hepatic stellate cells (HSCs), Kupffer cells (KCs), and recruited inflammatory cells.



Adapted from: Iredale, J.P. *J Clin Invest.* (2007) 117(3): 539-548.

- The key initiating and series of adaptive events that perpetuate this response, especially in humans, are still not well understood.
- The recent availability of bioprinted human liver tissue models that incorporate both parenchymal and non-parenchymal cells (NPCs) in a 3D context has created the opportunity to examine progressive liver injury in response to pro-fibrotic modulators and compounds.



Norona, L.M., *et al. Toxicological Sciences* (2016) 154(2): 354-367

Figure 1: 3D bioprinted tissue recapitulates the tissue-like density of native liver and exhibits key features for modeling fibrosis *in vitro*. (A) Depiction of a transverse cross-section of bioprinted liver tissue and gross tissue morphology, (B) H&E stained native liver and bioprinted liver. (C) The 3D context and key architectural relationships between parenchymal (HC) and NPCs (*i.e.*, HSCs and ECs) support normal liver function and maintain phenotypic features of cells for at least 42 days. CD31 (ECs), vimentin (HSCs), albumin (HCs), desmin (HSC quiescence marker), α-SMA (HSC activation marker).

Key Criteria for Modeling Fibrosis *In Vitro*

Multicellular and Tissue-like Architecture	• Fibrosis is a multifactorial process • Basic fibrogenic features are best interpreted in a 3D environment
Functional and Long-Lived	• Chronic low concentration exposure scenario
Preserves Phenotypic Features of HSCs	• Goal is to resolve some of the early compound-induced effects on fibrogenic outcome

Previously published studies (Norona *et al*., 2016), which showed progressive fibrotic injury in ExVive3D™ Liver tissues in response to model fibrogenic compounds, were expanded with the following objectives:

- **Assess tissue injury response to methotrexate with later initiation of injury in two independent tissue lots**
- **Assess tissue injury response over an extended exposure timeframe**
- **Evaluate the tissue injury response with the incorporation of Kupffer cells**

Methods

ExVive3D Liver Tissues with and without KCs were manufactured and provided by Organovo (San Diego, CA).

Maintenance and Compound Exposure. Tissues were maintained in ExVive3D Liver culture medium provided by Organovo (San Diego, CA) for 6 days prior to compound exposure. Exposure to vehicle (0.1% DMSO) or fibrogenic drug methotrexate (MTX; 0.01 to 10 µM) occurred daily for 14 to 28 days. Medium and tissue were collected on alternate treatment days and at the end of exposure, respectively.

Results

Cytokine abundance changes substantially over the first 3-7 days post-manufacture

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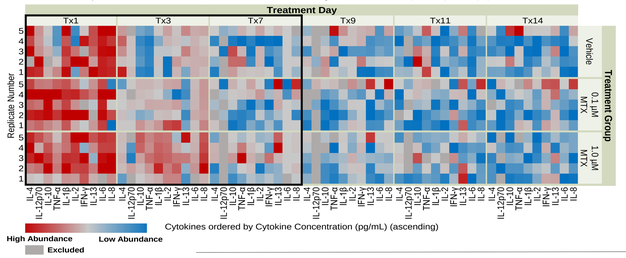

Figure 2: Heat map showing the overall abundance of cytokines released into the culture medium over 14 days of treatment. Tissues treated on post-manufacture Day 4 exhibited a surge in proinflammatory cytokine production at early timepoints and a rapid induction of collagen deposition within a 2 week timeframe (Norona *et al*., 2016). An approximate 70% reduction in baseline (vehicle) IL-8 and IL-1β to steady-state levels was observed within the first 10 days post-manufacture (black box), accompanied by a ~65% reduction in baseline LDH release (data not shown), suggesting that the initiation of compound treatment overlapped substantially with the post-manufacture wound healing response.

General injury profiles are consistent in two tissue lots treated with methotrexate for 14 days

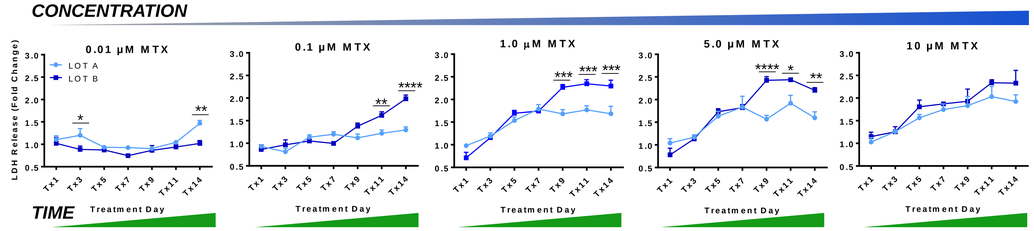

Figure 3: Methotrexate dose response in two lots of bioprinted tissue. Treated tissues exhibited similar trends in LDH release with an overall higher magnitude of response observed for LOT B at mid to late timepoints. Data represent the mean ± SEM; n = 5. Significance: Two-way ANOVA with post hoc Dunnett's multiple comparisons test (**p*<0.05, ***p*<0.01, ****p*<0.001, *****p*<0.0001).

Temporal differences in LDH and ALT suggest early hepatocellular injury

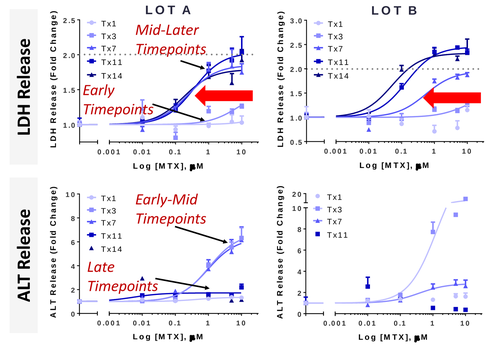

Figure 4: LDH and ALT dose response profiles for two lots of bioprinted tissue treated with MTX for 14 days. LDH release (general injury) and ALT (HC specific injury) were measured to understand the dynamics of injury over the course of MTX exposure. Shift in the dose-response profile (red arrows). Data represent the mean ± SEM; n = 5.

Methotrexate induces dose-dependent increases in collagen deposition

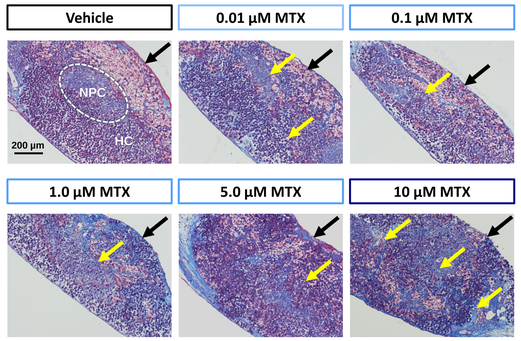

Figure 5: Trichrome-stained tissue sections from tissues treated with MTX (LOT B) for 14 days. Transverse cross-sections of tissue were stained to visualize collagen deposition (blue; yellow arrows), cytoplasm (pink/purple), and nuclei (dark purple). Black arrows (apical side of the tissue), non-parenchymal compartment (NPCs), and hepatocellular compartment (HC).

Expansion of exposure timeframe to methotrexate spans peak LDH release in treated tissues indicating mild injury

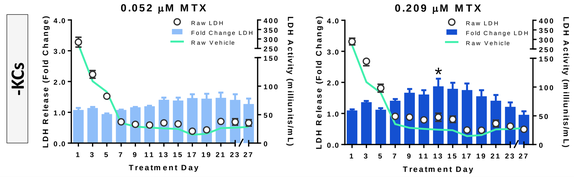

Figure 6: LDH release over 28 days of exposure to previously identified LC20 and LC50 MTX concentrations. During the first week of treatment, LDH release (open circles) gradually declined and approached steady state levels of production. A 1.5-2.0-fold increase in LDH was observed for Tx9-Tx19. Significance: One-way ANOVA (LDH) with post hoc Dunnett's multiple comparisons test (**p*<0.05, ***p*<0.01, ****p*<0.001, *****p*<0.0001).

Inclusion of KCs shortens the methotrexate-induced injury window and impacts biochemical endpoints

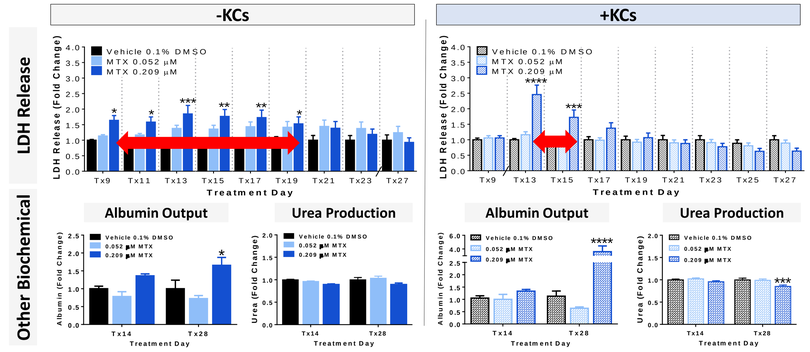

Figure 7: Impact of KCs on extended methotrexate exposure. The sustained release of LDH observed for the standard tissue model (-KCs) was attenuated with the incorporation of KCs. Increased albumin output was observed at Tx28 for 0.209 µM MTX with no change in urea production for the standard tissue model as expected. A significant increase in albumin and decrease in urea at Tx28 for 0.209 µM MTX suggests a perturbation of HC function with the incorporation of KCs. Data represent the mean ± SEM; n = 5-7. Significance: Two-way ANOVA with post hoc Dunnett's multiple comparisons test (**p*<0.05, ***p*<0.01, ****p*<0.001, *****p*<0.0001).

Initial histological assessment suggests KCs may limit the extent of collagen deposition during the early/mid treatment period

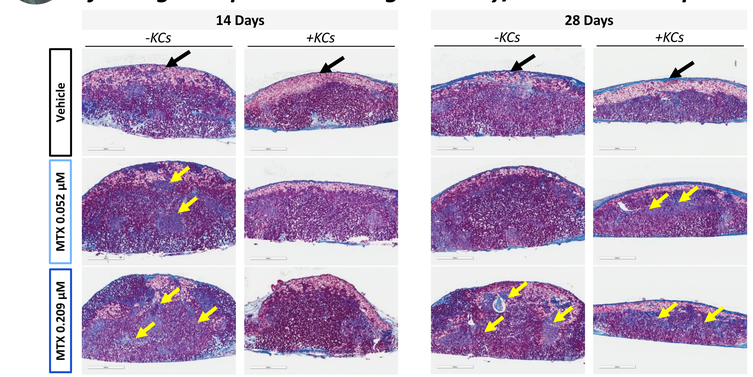

Figure 8: Trichrome-stained tissue sections from tissues treated with methotrexate in the presence and absence of KCs. Standard tissues (-KCs) exhibited dose-dependent increases in collagen deposition with MTX exposure. Continued exposure of +KC tissues to MTX resulted in dose-dependent increases in collagen deposition observed at the latest timepoint. Black arrows (apical side of the tissue), yellow arrows (areas of collagen deposition in blue).

Summary

- **ExVive3D™ bioprinted liver tissue represents a unique platform for measuring effects of repeated compound exposure.**
- **The tissue response to compound injury may be influenced significantly by cytokine milieu.**
- **Temporal patterns of LDH and ALT release are consistent across two independent tissue lots and suggest MTX-induced hepatocellular injury precedes general tissue injury.**
- **The incorporation of KCs shorten the general injury window and may attenuate the extent of collagen deposition during the early/mid exposure timeframe.**
- **This novel model system may lead to a better understanding of the early key events underlying chronic liver injury leading to fibrosis in humans and the classification of fibrogenic agents for risk assessment purposes.**

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