**SUPPORTING INFORMATION**

# Progressive Hypoxia-on-a-Chip: An *In Vitro* Oxygen Gradient Model Capturing the Effects of Hypoxia on Primary Hepatocytes in Health and Disease

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**Supplementary Results**

**Cytotoxicity of sulfite and cobalt in well plate hepatocyte cultures**

Cytotoxicity of sulfite is led by ROS accumulation that results in cell death through protein and DNA damage(Hancock, Desikan, & Neill, 2001; Jiang et al., 2011; Niknahad & O'Brien, 2008). Sulfite that is not converted to other metabolites by sulfite oxidase causes the loss of antioxidants, resulting in cytotoxicity(Jiang et al., 2011; Niknahad & O'Brien, 2008). Metal chelators such as cobalt play a role in preventing the cytotoxicity of sulfite. With this understanding, we investigated the cytotoxicity of sulfite and cobalt on hepatocytes. Primary hepatocytes cultured in well plates were treated with media containing 0% sulfite/0 µM cobalt, 0.25% sulfite/25 µM cobalt, 0.5% sulfite/50 µM cobalt, 0.75% sulfite/75 µM cobalt, 1% sulfite/100 µM cobalt, 100 µM cobalt, 1% sulfite, and 95% $N_2$/5% $CO_2$ for 2, 4, 8, and 24 hours. Simultaneously, cells were exposed to oxygen-free media supplemented with sulfite and/or cobalt or nitrogen gas for 2 hours and then cultured in fresh DMEM media for an additional 24 hours to assess the cell recovery. After these treatments, the cell viability was quantified via TMRM staining among different experimental groups.

Hepatocytes exposed to 100 µM cobalt maintained full cell viability over the course of 24 hours, similar to the control group (0% sulfite/0 µM cobalt) (Figure S2). On the contrary, hepatocytes exposed to 0.5% sulfite/50 µM cobalt, 0.75% sulfite/75 µM cobalt, and 1% sulfite/100 µM cobalt for 2 hours maintained cell viability of over 85% and the viability for these groups gradually decreased to approximately 0% at the end of 24 hours, whereas the cell viability of hepatocytes exposed to 0.25% sulfite/25 µM cobalt only decreased to 70% (Figure S2). Over the 2 to 24 hour time frame, a gradual decrease in cell viability from 90% to less than 10% was observed for the hepatocytes exposed to 95% $N_2$/5% $CO_2$. This suggests that the loss in cell viability was caused by oxygen deprivation rather than toxicity as $N_2$ and $CO_2$ are non-toxic to cells. The viability of hepatocytes exposed to all conditions, except for 1% sulfite, was recovered (over 90%) following the 24-hour incubation in fresh media. In the case of the 1% sulfite only solution, its cytotoxicity may be led by ROS accumulation and also accompanied with depletion of an antioxidant

defense system. In addition, the absence of metal chelators might promote cell death. These results indicate that cell death in the mixed sulfite and cobalt solutions might be caused by oxygen depletion rather than cytotoxicity of sulfite or cobalt.

Together, the mixed 1% sulfite/100 µM cobalt media, which was used to create the severe hypoxia condition in the MPOC device, did not result in significant toxicity for the first 2 hours.

**Generation of the oxygen gradient in the MPOC device.**

The oxygen level range observed along the liver sinusoid *in vivo* is 3.9% - 8.6% $O_2$(Brooks, Eastwood, Beckingham, & Girling, 2004; Brooks, Hammond, Girling, & Beckingham, 2007; Kietzmann, 2017; M, 1996; Wolfle, Schmidt, & Jungermann, 1983). In order to achieve this normoxic gradient for modeling tissue physiology, we tested various concentrations of chemical scavengers in the MPOC. We obtained an oxygen gradient of 11.2 ± 0.8% - 6.9 ± 0.7% inside the device channels as measured by the VisiSens oxygen detection system when we used culture media supplemented with 0.13% sulfite and 13 µM cobalt in one inlet and regular culture media with no sulfite or cobalt in the other inlet(Beyza Bulutoglu, 2019). This established oxygen concentration range is closer to the physiological oxygen gradient of 3.9%-8.6% along the liver sinusoid(Brooks et al., 2004; Brooks et al., 2007; Wolfle et al., 1983). A normoxic gradient of 3.9% - 8.6% can be achieved by mixing a concentration of sulfite and cobalt less than 0.25%/25 µM and another concentration close to 0% sulfite/0 µM cobalt.

**Supplementary Figures**

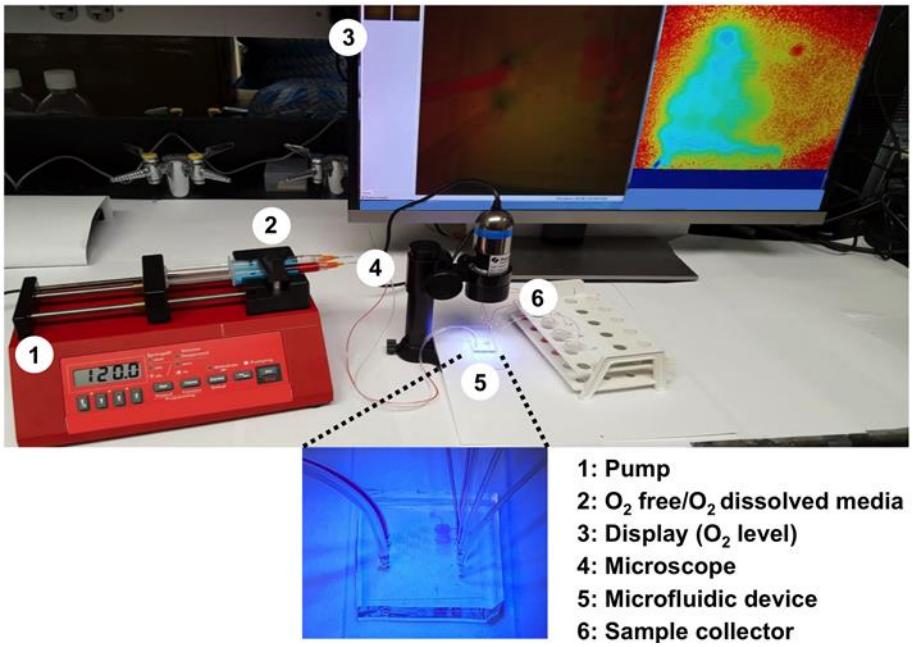


**Figure S1**. The hypoxia gradient microfluidic device and the oxygen detection system. The hypoxia on-a-chip was connected to a syringe pump and five outlets of the device were connected to sample collection tubes. Oxygen levels were detected by a PreSens oxygen measurement system. Oxygen gradients were created by mixing 0% sulfite/0 µM cobalt ($O_2$ dissolved media) and 1% sulfite/100 µM cobalt ($O_2$ free media).

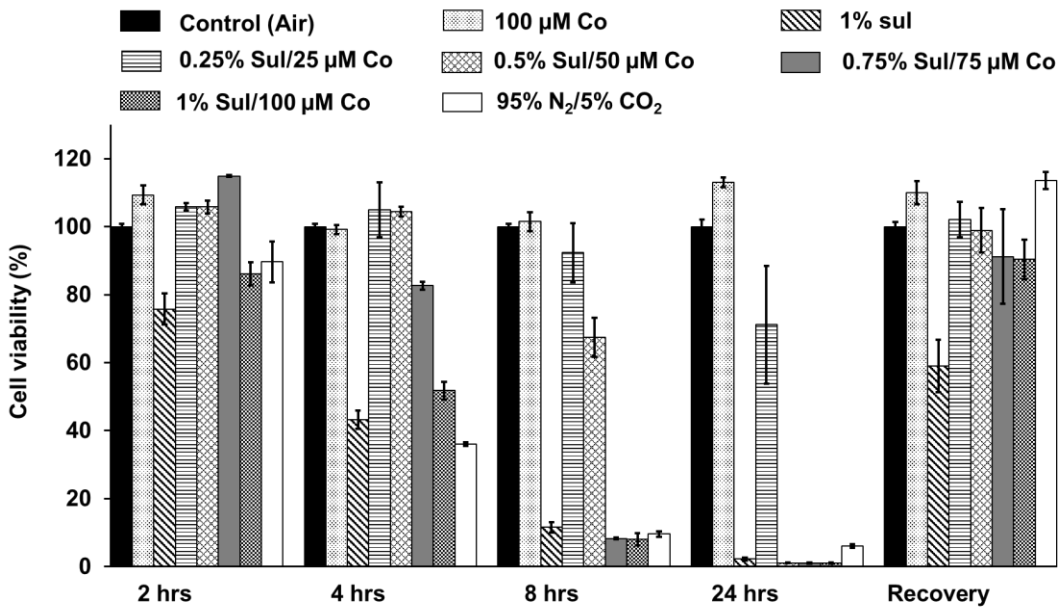


**Figure S2.** Cytotoxicity of sulfite and cobalt on primary rat hepatocytes. Primary rat hepatocytes were exposed to media containing: 0% sulfite/0 µM cobalt, 0.25% sulfite/25 µM cobalt, 0.5% sulfite/50 µM cobalt, 0.75% sulfite/75 µM cobalt, 1% sulfite/100 µM cobalt, 100 µM cobalt, 1% sulfite, and 95% $N_2$/5% $CO_2$ for 2, 4, 8, and 24 hours. In addition, cells exposed to these conditions for 2 hours were cultured in the fresh media for another 24 hours to assess cell recovery. At the end of each experiment, cell viability was measured via TMRM staining. All data were obtained from at least three different hepatocyte isolations and are reported as the mean ± standard error of the mean.

**Supplementary Tables**

**Table S1**. Oxygen concentrations of sulfite and cobalt solutions in water.

| Solution | $O_2$ Concentration (%)* |
|---|---|
| 0% sulfite/0μM cobalt | 15.9 ± 1.1 |
| 0.25% sulfite/25μM cobalt | 1.6 ± 1.0 |
| 0.50% sulfite/50μM cobalt | 0.7 ± 0.5 |
| 0.75% sulfite/75μM cobalt | 0.3 ± 0.2 |
| 1.0% sulfite/100μM cobalt | 0.0 ± 0.0 |
| 100μM cobalt | 14.8 ± 0.2 |
| 1% sulfite | 0.0 ± 0.0 |
| 95% $N_2$/5% $CO_2$ | 0.0 ± 0.0 |

* The values are reported as mean ± standard error of mean (n=3).

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