

MRI-based detection of extracellular changes in the hepatic sinusoid in a rat model of non-alcoholic steatohepatitis

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Introduction: The goal of this work was to detect changes to the extracellular matrix (ECM) of the diseased liver in vivo using MRI. Chronic liver disease is marked by dramatic changes to the ECM surrounding the hepatic sinusoid (HS) (1). With the incidence of liver diseases such as non-alcoholic fatty liver disease (NAFLD) increasing in both the general and pediatric populations (2), a method for early detection of structural and functional changes to the liver could be a vital tool in the clinic. It was previously shown that the ECM in the kidney glomerulus can be detected using MRI after intravenous (IV) injection of a cationized form of the superparamagnetic iron-storage protein ferritin (3). This technique is based on the electrostatic binding between the cationic ferritin (CF) and the anionic proteoglycans of the ECM. Here we show that intravenously injected CF binds specifically to the ECM surrounding the HS and is eventually internalized by hepatocytes, Kupffer's cells, and endothelia. Furthermore, we show that IV injection of CF in a rat model of non-alcoholic steatohepatitis (NASH), an advanced stage of NAFLD, yields unique labeling in the liver compared to control animals and that this difference is detectable using MRI. These results suggest that MRI after the IV injection of CF may be used as a non-invasive tool measure changes in ECM in chronic liver disease. **Methods:** *Synthesis of Cationic Ferritin:* Cationic ferritin (CF) was synthesized from horse spleen ferritin per Danon et al. (4). *Disease Model:* 18 male Sprague-Dawley rats were used for the dietary methionine choline deficient model of NASH. Nine rats ate a methionine choline deficient (MCD) diet for 14 weeks to establish NASH. The other nine ate a control diet of the same composition supplemented with methionine+choline for 14 weeks. Serum alanine transaminase (ALT) was measured via photometric absorbance in 6 MCD and 6 control animals to determine disease maturity. All experiments took place after serum ALT indicated disease. *Animal Preparation:* Six MCD and six control rats were imaged at 7T prior to CF injection. Rats then received 5.75 mg/100 g of CF in PBS, IV, in three doses spaced by 1.5 hrs. The rats were imaged again at 7T 1.5 hrs after the final injection of CF and then sacrificed via transcardial perfusion of PBS and formalin three hours after the final injection of CF. Livers were resected and prepared for IHC and ex vivo MRI. Three MCD rats and three control rats were sacrificed prior to CF injection. Their livers were prepared for IHC and ex vivo MRI. A separate group of three healthy rats were administered the same dose of CF as above and sacrificed 1.5 hrs after the final injection of CF. Their livers were prepared for IHC. *MRI:* For in vivo MRI, rats were anesthetized with isoflurane and imaged at 7T using a series of 5 T_2^* -weighted 2D-gradient echo sequences. TE was incremented from 4 ms to 8 ms with TR=60 ms. In vivo images were collected with a resolution of 234x234x1000 μ m. Perfused ex vivo livers were imaged at 7T using a series of 13 T_2^* -weighted 2D-gradient echo sequences. TE was incremented by 0.5 ms from 2.5 ms to 8.5 ms with TR=56 ms. Ex vivo images were collected with a resolution of 328x328x1000 μ m. All MRI was done on a Bruker 7T/35 scanner. *Histology:* Immunohistochemistry was performed on excised livers to determine the distribution of injected CF. Tissue were sectioned, washed, blocked, and incubated in chicken anti-fibronectin and rabbit anti-horse ferritin primary antibodies and Alexa488 goat anti-chicken and Alexa594 goat anti-rabbit secondary antibodies. Sections were then incubated with DAPI, mounted, and imaged on Zeiss 710 laser scanning confocal microscope. *Data Analysis:* In vivo and ex vivo T_2^* values for each voxel were calculated in MATLAB based on the five in vivo TE values and 13 ex vivo TE values collected pre- and post-injection of CF. Statistical analyses were run as two-sample, two-tailed Student's t-tests ($\alpha=0.05$). *Results:* Serum measurements of ALT suggest that the dietary NASH model was established at 14 weeks in MCD rats (Fig. 1). Establishment of disease marked the beginning of all other experiments. The uptake of CF in MCD livers 1.5 hrs after injection is lower than the uptake of CF in control livers. This difference is detectable with in vivo and ex vivo T_2^* measurements (Figs. 2 and 3), normal T_2^* -weighted gradient echo imaging (not shown), and confocal microscopy (Fig. 4). Ex vivo MRI was used to confirm the accuracy of in vivo T_2^* changes with TE values much shorter than T_2^* and 13 echo times. Furthermore, confocal imaging shows that CF is specifically bound to the ECM surrounding the HS 1.5 hrs after injection (when our in vivo MRI was done) and is eventually (3 hours after final CF injection) internalized by hepatocytes, Kupffer's cells, and endothelia (Fig. 5), suggesting that 1.5 hours after CF injection is an ideal time to specifically detect ECM surrounding the HS with MRI. **Conclusions:** The goal of this work was to detect changes to the ECM of the HS due to chronic liver disease. In vivo T_2^* measurements in control rats show a 50% reduction in T_2^* . Confocal microscopy suggests that this T_2^* change is due to CF-labeling in the liver. No such T_2^* changes were seen in MCD rats (rats with chronic liver disease). Microscopy also suggests that this is due to reduced CF-labeling in the diseased livers. The reduced CF-labeling in MCD rats is likely due to structural changes to the ECM that are known to occur during development of chronic liver disease (1). **These results indicate that IV injection of CF may be used to detect ECM-specific changes in chronic liver diseases such as NAFLD.**

References: (1) Bedossa, P et al. *J Path.* 200, 2003 (2) Erickson, S *Clin. J. Lipid Res.* 50, 2009 (3) Bennett, KM et al. *Magn Reson Med.* 60, 2008 (4) Danon, D et al. *J Ultrastruct Res.* 38, 1972

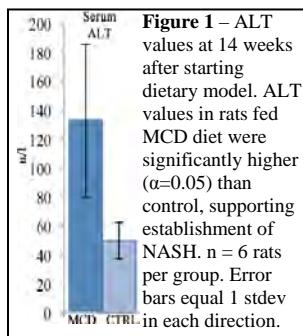


Figure 1 – ALT values at 14 weeks after starting dietary model. ALT values in rats fed MCD diet were significantly higher ($\alpha=0.05$) than control, supporting establishment of NASH. n = 6 rats per group. Error bars equal 1 stdev in each direction.

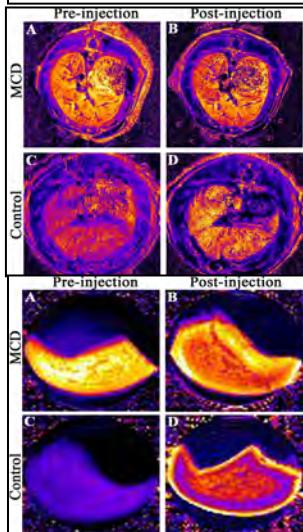


Figure 2 – In vivo T_2^* maps. A significant ($\alpha=0.05$) ~50% reduction in T_2^* was observed in livers of control rats after CF injection. T_2^* in livers from MCD rats did not change significantly after CF injection ($\alpha=0.05$). n=6 rats per group. Error bars = 1 stdev in each direction.

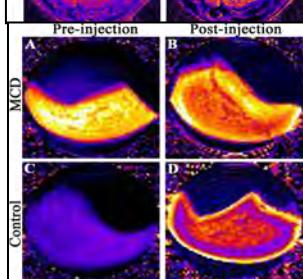


Figure 3 – Ex vivo T_2^* maps. A significant ($\alpha=0.05$) ~50% reduction in T_2^* was seen in control livers after CF injection. T_2^* in MCD livers did not change significantly after injection ($\alpha=0.05$). Post-injection n=6 rats, pre-injection n=3 rats. Error bars = 1 stdev in each direction.

Figure 4 – Confocal imaging of MCD and control livers pre- and post-injection of CF. Substantial fluorescence from CF (red) can be seen in post-injection controls compared to pre-injection controls. Less CF-labeling is seen post-injection in MCD livers. ECM (green) pattern in MCD livers is different from control livers due to tissue displacement by lipid droplets. Scale bar=20 μ m. Blue fluorescence is DAPI.

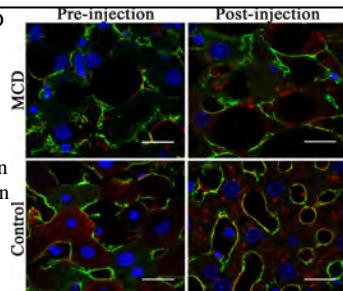


Figure 5 – Confocal imaging of healthy rat hepatic sinusoids at 1.5 and 3 hours post-injection of CF. CF (red) is specifically labeled to the ECM (green) 1.5 hours after injection. 3 hours after injection, CF has been internalized into hepatocytes, Kupffer's cells, and endothelia. Scale bar=20 μ m. Blue fluorescence is DAPI.

