Published in final edited form as:

J Magn Reson Imaging. 2015 February ; 41(2): 347–353. doi:10.1002/jmri.24616.

Hepatic Fat During Fasting and Refeeding by MRI Fat Quantification

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Abstract

Purpose—To explore the sensitivity of high-field small animal magnetic resonance imaging to dynamic changes in fat content in the liver and to characterize the effect of prandial state on imaging studies of hepatic fat.

Materials and Methods—A total of 3 time-points were acquired using asymmetric spin-echo acquisitions for 12 mice with 24 hour spacing. After the first scan, half of the cohort was placed on a water-only diet. The second half of the cohort continued to have access to their high-fat chow. The scans were repeated after 24 hours. All animals were then returned to the high-fat diet, and the scans were again repeated after 24 hours. Fat fraction maps were computed using previously described methods. Regions of interests were manually drawn in the livers, and the patterns of the two groups over time were compared.

Results—Five out of six of the animals in the starved group showed an increase in hepatic fat fraction during the fasting period (average increase 0.54 ± 0.48), which decreased on refeeding. ANOVA analysis indicated that the results significantly depended on both the group and the time-point ($p=0.003$).

Conclusion—Fat-water imaging methods are able to measure hepatic fat changes caused by short-term dietary perturbations.

Keywords

hepatic fat prandial state; fasting refeeding; in vivo mouse imaging

Introduction

Non-Alcoholic Fatty Liver Disease (NAFLD) is the most common cause of chronic liver disease in the United States(1,2). It is characterized by the accumulation of fatty acids in the liver due to causes other than alcohol and toxins(3–5). NAFLD affects about 20% of the

general population, and about 80% of people with body mass index (BMI) greater than 30(5). It can lead to fibrosis(6), liver failure and hepatocellular carcinoma(2). Due to the large disease burden, it is important to be able to predict progression of the disease, particularly using non-invasive methods(6,7), with the aim of decreasing mortality due to liver disease(8).

Animal models such as *ob/ob* knockout mice, or mice fed choline/methionine deficient diets(9) have been used to observe the effects of various treatments and physiological perturbations on hepatic fat. However, past methods of measuring hepatic fat in small animals have been insufficient for making serial observations of hepatic fat through a period of fasting and refeeding. Biopsy is prone to sampling error(10), and most non-invasive imaging modalities, besides magnetic resonance spectroscopy and imaging, are not chemically quantitative(5). Only recently, MRI methods to quantify fat have been developed. The IDEAL method(11,12) has been validated for single measurements of hepatic fat(13), but the utility of imaging methods in monitoring changes in hepatic fat has not been shown.

The primary purpose of this paper is to explore the sensitivity of 7T small animal imaging methods(14,15) to dynamic changes in fat content in the liver. A secondary purpose of this paper is to characterize the effect of prandial state on imaging studies of hepatic fat, so as to help better plan future imaging studies.

Methods

Twelve high-fat diet fed 30–36 week-old male C57Bl/6 mice were fed a high-fat diet starting after weaning. We housed the animals in metabolic cages (Nalgene, Pennfield, NY). These cages ensure strict fasting by allowing removal of access to all food, leaving only the plastic and metal of the cage in contact with the animal living area. We found in preliminary studies not reported here that the starvation state was not properly achieved without the use of this special cage. The mice were first habituated to the cage environment by being housed in these cages for 3–4 days prior to the experiment. During the experiment, the mice were housed individually in the metabolic cages, some with access to food, and some without, as described further below, but water was provided to the animals at all times. All procedures were performed with approval from the Institutional Animal Care and Use Committee.

All animals were scanned using a set of 2-D asymmetric spin echo scans under isoflurane anesthesia on a Bruker BioSpec 7T/30cm scanner with respiratory gating. A 20% Intralipid (Baxter Healthcare, Deerfield, IL) phantom was included in the field-of-view in each image volume. The asymmetry times were set to acquire three image volumes per animal with fat/water phase differences of $\pi/6$, $5\pi/6$, and $3\pi/2$, which corresponded to 7.93e-5, 3.96e-4 and 7.14e-4 second delays between the Hahn echo and gradient echo(16). A nominal echo time of 10 ms and repetition time of 1500 ms was used, with a resolution of 0.2×0.2×1 mm. The oxygen flow rate was held constant at 2L/min and isoflurane concentration delivered to the animal was controlled to maintain a breathing rate of about 30 breaths/minute. The temperature of the animal was maintained automatically using a feedback control, which was set to maintain the rectal temperature of the mouse at 34°C.

After the first scan, half of the cohort (“fasted” group) of animals was placed on a water-only diet, with no chow available, for 24 hours. The second half of the cohort (“fed” group) continued to have access to their high-fat chow. The set of three scans described above were repeated after 24 hours. Finally, all animals were returned to the high-fat diet, and the scans were again repeated after 24 hours. A total of 3 time-points were collected for each mouse with 24 hour spacing. The animals were also weighed before each time-point.

Fat-water decomposition at each time-point was performed using FLAWLESS(14) to prevent swapping errors. The field maps were corrected using REFINED(15) to automatically correct swaps that might have arisen. A fat fraction map was computed, as described previously(17):

$$Fat\ Fraction = \frac{|\rho_{fat}|}{|\rho_{water}| + |\rho_{fat}|} \quad [1]$$

where each ρ represents the proton density of that species. A single, “best” slice was chosen by manually searching through the source data images and the ρ_{water} parameter maps, looking for a minimal amount of motion artifact, and a minimum number of swaps. This slice also had to include a part of the phantom in the field-of-view. A region of interest (ROI) was manually placed in the liver, using the ρ_{water} image as a reference. The ROI was drawn to be as inclusive of the liver as possible in that image. The mean and variance of this region in the fat fraction map were computed and were taken to be indicative of the baseline fat content of the liver. An additional ROI was placed in the phantom. The mean and variance of this region in the fat fraction map were also computed. The hepatic fat fraction was normalized to the measured phantom fat fraction using the following equation:

$$Hepatic\ Fat\ Fraction_{normalized} = Hepatic\ Fat\ Fraction_{original} \frac{Phantom\ Fat\ Fraction_{known}}{Phantom\ Fat\ Fraction_{measured}} \quad [2]$$

where $Phantom\ Fat\ Fraction_{known}$ was 0.2, the concentration of fat in the Intralipid. Computation of the fat fraction and manual analysis was performed in software developed in-house in MATLAB (MathWorks, Inc., Natick, MA). Normalization of the fat fraction and further analysis of the aggregate results were performed in LibreOffice Calc (The Document Foundation, Berlin, Germany). Both were performed on a 3.2GHz computer with 8GB of RAM (iBUYPOWER, Inc., Los Angeles, CA).

To examine the variability of the measurements due to placement of the ROI, we chose one of the animals to explore in more detail. We quantified the variability of the measurement due to human variation in manual ROI placement, by placing a second ROI in both the liver and the phantom in the same slice as mentioned before. To examine the slice-to-slice variation, two additional slices were chosen per time-point, and ROIs were placed in the liver and phantom.

After aggregation of the normalized hepatic fat fraction and weight of each animal at each time-point, we computed change during the “fasting” period for both the fasted and fed groups using the following calculation:

$$\Delta \text{Hepatic Fat} = \frac{\text{Fat Fraction}_{\text{Fasting}} - \text{Fat Fraction}_{\text{Baseline}}}{\text{Fat Fraction}_{\text{Baseline}}} \quad [3]$$

A similar analysis was performed with a relaxation-compensated fat fraction (RCFF)(18), which was calculated as previously described, and as follows:

$$RCFF = \frac{\frac{|\rho_{fat}|}{(1 - e^{-TR/T_1^{fat}})e^{-TE/T_2^{fat}}}}{\frac{|\rho_{water}|}{(1 - e^{-TR/T_1^{water}})e^{-TE/T_2^{water}}} + \frac{|\rho_{fat}|}{(1 - e^{-TR/T_1^{fat}})e^{-TE/T_2^{fat}}}} \quad [4]$$

The T1 and T2 values that were used in the description of RCFF(18) were used here. The nominal TR and TE values, as delineated above, were also used in the calculation.

An unpaired, Welch's t-test(19) was performed to test for significance of the differences between the two groups of animals. A double-tailed t-test was used to compute a p-value for each of the following quantities: initial weight, initial hepatic fraction, and initial hepatic RCFF. A single-tailed t-test was used to compute a p-value to compare the weight change between baseline and fasting time-points. Finally, a two-way ANOVA was computed to test if MRI can be used to detect changes in hepatic fat caused by dietary perturbations.

Results

Figure 1 shows the mass of each animal during the experiments. The starting mass of the Starved group was 40.3 ± 4.8 g, while the starting mass of the Fed group was 40.0 ± 1.2 g, which were not significantly different ($p = 0.91$). The Starved group lost 3.3g during the fasting time, while the Fed group lost 0.7g during the same period. During the refeeding time, the Starved group gained 0.8g, while the Fed group lost 0.3g. Overall, the Fed group lost 1g, while the Starved group lost 2.44g. These patterns imply that the fasting state was achieved during the fasting period of the experiment, but there is also some weight loss associated with simply going through the experiment, perhaps due to the physiological stress or due to lack of access to food during the scan times.

Figure 2 shows a representative sample of three source images that were acquired. Proper respiratory gating allowed the ghosting due to motion to be minimized, though this was very difficult to avoid around the lungs. The scans were arranged to include the phantom in the same field-of-view as a part of the liver.

Figure 3 shows the output of FLAWLESS(14) plus REFINED(15) processing. The use of these algorithms greatly decreased the number of fat-water swaps appearing in the output parameter maps. These output images were used for manual ROI placement, as described above.

Figure 4 shows the results of the ROI analysis. The mean of the phantom measurements for this set of measurements was 0.27 ± 0.05 , where the 0.05 was computed as the mean of the standard deviations within each phantom ROI. The square root of the mean squared (RMS) error between two ROIs placed in the same slice, after normalization to the phantom, was

0.005. This number reflects the square root of the squared difference between the means of the ROIs. The RMS error between the original measurement and the average of the three measurements was 0.021. This error can be considered to be small, because it was smaller than the mean variability within the phantom ROI, i.e., 0.05.

Figure 5 shows the normalized hepatic fat fractions of the fasting-refeeding study, by animal. The average value of the fat fraction in the phantom ROI, averaged across all of the trials, was 0.290 ± 0.042 . After normalization to the phantom measurement, the mean starting hepatic fat fraction of the Starve group was 0.146 ± 0.055 , while that of the Fed group was 0.200 ± 0.107 . These were not significantly different ($p = 0.47$). The Fed group did not show a consistent pattern in hepatic fat fraction, with some increasing, some decreasing, and some staying the same. Five out of six of the animals in the Starve group showed an increase in hepatic fat fraction during the fasting period, which at least partially abated on refeeding. Using RCFF instead of the fat fraction, the mean of the phantom was 0.14 ± 0.02 . After normalization in the phantom, the mean starting hepatic fat fraction was 0.14 ± 0.06 for the Starve group and 0.21 ± 0.14 for the Fed group. These were not significantly different ($p = 0.63$).

Figure 6 shows the average change in hepatic fat fraction between the Baseline and Fasting time-points, as computed by Equation 3 and averaged over each group. The Starve group had a larger (Hepatic Fat = 0.54 ± 0.48) increase during this time than the Fed group (Hepatic Fat = -0.08 ± 0.24). With RCFF, after normalization, the mean change in hepatic fat was 0.66 ± 0.58 for the Starve group and -0.09 ± 0.28 for the Fed group. A computation similar to the Hepatic Fat (Equation 3) was additionally performed between the fasting and refeeding time-points, rather than between the baseline and fasting time-points. During this period, the Starve group lost more hepatic fat (-0.19 ± 0.16) than did the Fed group (-0.07 ± 0.19). Table 1 shows the result of a two-way ANOVA. The p-value of the Interaction term was 0.003, which indicates that the results significantly depended on both the group and the time-point. This further indicates that our experiment was able to measure hepatic fat changes caused by dietary perturbations.

Discussion

In this work, we showed evidence that MRI methods can be used to monitor changes in hepatic fat in mice. The result of a two-way ANOVA indicated that we were able to measure changes elicited by a dietary model ($p = 0.003$).

Long-term feeding with a high-fat diet is associated with increases liver fat and the development of NAFLD(20). However, the effect of short-term refeeding with high-fat food after fasting is more complicated. A high carbohydrate meal has been shown to induce *de novo* lipogenesis(21), and either a high carbohydrate or a high fat meal can increase postprandial lipemia(22). In the liver, a high carbohydrate meal after fasting causes an increase in hepatic fat compared to baseline (after about 72 hours in rats)(23). The addition of a small amount of fat to this diet prevents this increase. The specific physiology of experimental case has not been studied to our knowledge.

There was some phenotypic variability in our study populations: one of the six mice in the Starve group did not show the same pattern as the others upon fasting. This mouse also lost the most mass during the fasting period (a full gram more than the average of 3.2g lost), so it is possible that the peak seen after a day of fasting was missed (see reference (24) for more details). However, the Fed group showed an inconsistent pattern in liver fat, which means that there are possible confounds in this experiment. First, it is possible to attribute the variation in the Fed group to the prandial state of the particular mice at the time of each measurement, since access to food does not guarantee that each mouse is in the same prandial state. Second, isoflurane anesthesia can sometimes cause hepatic necrosis(25), which might decrease the apparent fat fraction by increasing the water content with the inflammatory response. The incidence of this necrosis is higher in fasting animals, which might also explain the phenotype of the Starve group mouse that did not follow the trend of the other mice.

In a previous study, which was done by sacrificing lean mice, rather than by making serial measurements, a 69% increase in hepatic fat was found after a day of fasting(24), as compared to our 52% increase in fasting obese mice, without relaxation compensation. After nominal relaxation compensation (i.e., $TR=1.5s$), we found a 63% increase in hepatic fraction with fasting, though the significance of this difference ($p = 0.18$) remained about the same as the significance without relaxation compensation. Ideally, stricter significance criteria should be used for studies that would quantify physiology, but the number of required subjects that would have been necessary to reach these stricter criteria would have been prohibitively expensive for this preliminary imaging study.

The phantom data demonstrates the consistent performance of the methods used in this work, because the variability in the phantom measurements between different trials was about the same as the variability within each phantom ROI. Since this variability within each ROI can likely be attributed to the noise and other artifacts of the imaging, it can be considered to be the measurement error. Furthermore, since the variation across ROIs was smaller than the mean variation within the phantom ROI, the placement of the ROI can be viewed as a minor error.

However, measurement error and noise alone do not account for a lack of accuracy in the phantom measurements. First, as has been previously discussed(26), accuracy is affected by the spectral modeling. However, spectral modeling requires additional scans, and since this is a study of sensitivity, rather than accuracy of these imaging methods, we did not believe the additional accuracy (a 3% difference in correlation coefficient) to be worth the additional scan time. The biggest source of error in the phantom measurements was relaxation compensation. The effect of T2 can be greatly mitigated by using the RCFF(18). However the overestimation of fat due to incomplete T1 relaxation is much more difficult to remove in animals studies. Previous animals studies have decreased the effect of T1 relaxation by using a small (5°) flip angle(13,27,28,29). However, this decreases the signal-to-noise ratio of the source images, which is not desirable. Another approach is to use the RCFF, which works well when phantoms are scanned independently of the animal, and when the nominal TR is the same as the true TR. However, when the scans are gated by respiration, the true TR depends on the respiratory rate. Assuming too short of a TR can over-correct the

problem, leading to an underestimate of the amount of fat in the sample. Since we attempted to keep the breathing rate at 30 breaths/minute, we estimated the true TR to be 3s. This improved the estimate in the phantom to 0.16, but since the true TR can vary with each breath, the effect of T1 cannot be completely removed without individually monitoring when each line of k-space was acquired, which would require a much more complicated acquisition scheme that we felt was outside the scope of this work. The use of the spin-echoes minimized the effect of T2* (12) on our data, in that T2† was very small, due to very short delays between the Hahn and gradient echoes. The T2 component was present, but was a smaller source of variation than the T1 recovery.

Since the fat fraction of the phantom was known, we used the phantom measurement to normalize the hepatic measurements, with the expectation that this would help to mitigate the measurement-to-measurement variation due to the variable breathing rate. In some of the trials, we included a second phantom with 0% fat content. Prior to normalization, the fat content in this phantom was 0.107 ± 0.022 . After normalization to the 20% fat phantom, the mean fat content of the second phantom was 0.067 ± 0.011 . Though this shows a bias in the measurement, it also indicates the precision of the measurement after normalization. Performing an experiment with a very long TR (about 15s) enables the accurate quantification, with no bias, of the fat content of a phantom, but this is impractical to do *in vivo*.

The advantage of imaging methods over destructive methods is that the same animal can be serially monitored, and the effects of perturbations can be monitored as they happen. However, there are many factors, such as prandial state, that can affect the fat content of the liver. We were able to show in this work that small animal imaging is sufficiently sensitive to measure these dynamic changes in liver fat content.

Acknowledgments

The authors would like to thank Mohammed Qutaish and Bernadette Erokwu, for their help with the experiments, Bakeerathan Gunaratnam and Hal Haller for their discussions on statistics, and Suguna Narayan for her editorial help with this article. Sreenath Narayan's effort was supported by in part by Award Number F30DK082132 from the National Institute of Diabetes and Digestive and Kidney Diseases, and in part by NIH grant T32GM07250 to the Case MSTP from the National Institute of General Medical Sciences. The work was supported in part by an Ohio Biomedical Research and Technology Transfer award, "The Biomedical Structure, Functional and Molecular Imaging Enterprise," and in part by R01-EB004070. This investigation was conducted in a facility constructed with support from Research Facilities Improvement Program Grant Number C06 RR12463-01 from the National Center for Research Resources, National Institutes of Health. The content is solely the responsibility of the authors and does not necessarily represent the official views of the NIDDK, the NIGMS, NIBIB, the NCRR, or the NIH.

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Figure 1.

Mass Patterns Imply Starve Group was in the Fasting State. The mass of each animal is plotted a function of time. The Starve group (top) lost more mass during the fasting period than the Fed group (bottom), and gained mass during the refeeding period. The mass of the Fed group also decreased during the experiment, but less than the Starve group, which implies that the Starve mice were in the fasting state for the second scan.

$\pi/6$ Source Image

$5\pi/6$ Source Image

$3\pi/2$ Source Image

Figure 2.

Example Source Images for FLAWLESS Decomposition with Minor Respiratory Motion Artifact. The three asymmetric spin echo (RARE, nominal TE = 10ms, nominal TR = 1500ms) raw data images are shown, after cropping out air pixels around the edges, with varying phase differences between the fat and water protons (as labeled). Although respiratory gating was used to minimize ghosting artifact, some motion artifact was unavoidable near the lungs and heart (arrows). Fat and water content at each pixel was estimated from these images.

Figure 3.

Example Quantitative Parameter Maps from FLAWLESS Decomposition. Examples of the three parameter map outputs of FLAWLESS are shown. A single slice was chosen from the middle of each image volume by a human operator, by observing the source images for a lack of motion artifact, the field map for a lack of discontinuities indicating swapped areas, and the water parameter map for further evidence of swaps. Using a water map as a reference, ROIs were drawn in the liver and the phantom, taking care to avoid any residual motion artifact and the edges of the body of interest. The mean of these ROIs in the fat fraction map was computed.

Figure 4.

Variability due to ROI Placement was Usually Lower than Standard Deviation of Phantom Measurements. In a test animal, two additional ROIs (total of 3) were placed in separate slices of the image volume. The average and standard deviations are shown for each time-point in the bar graph above. The square root of the mean of squared error between the original measurement and the average of the three measurements was 0.021, which was less than the mean variability within the phantom ROIs.

Figure 5.

Individual Hepatic Fat Fractions. The normalized hepatic fat fraction for each animal is plotted as a function of time. The fat fraction for the Starve group (top) increased during the fasting period for 5/6 of the animals, and abated during the refeeding period. The Fed group showed no consistent pattern. An increase with fasting is consistent past studies of physiology.

Figure 6.

Change in Hepatic Fat Fraction Between Baseline and Fasting Time-Points. The Starve group had a mean increase of 0.52 ± 0.58 (Equation 3), while the Fed group had a mean increase of 0.003 ± 0.26 . The Starve group increased more than the Fed group ($p = 0.18$).

Table 1**Two-way ANOVA Table**

Source	DF	SS	MS	F	P
Group	1	0.0048078	0.0048078	2.29	0.145
Time-point	1	0.0091825	0.0091825	4.38	0.049
Interaction	1	0.0232758	0.0232758	11.11	0.003
Error	20	0.0419050	0.0020952		
Total	23	0.0791711			
S = 0.04577		R-Sq = 47.07%		R-Sq(adj) = 39.13%	

P-value of Interaction term shows that hepatic fat is significantly dependent on both group and time-point, i.e., that MRI is capable of measuring hepatic changes associated with dietary perturbations