

MYOCARDIAL ISCHEMIA AND INFARCTION

Assessment of Reperfused Myocardial Infarction in the Hyper-Acute Phase with Delayed Enhancement Magnetic Resonance Imaging

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ABSTRACT

Purpose: This study intends to investigate the earliest point which the reperfused infarct size can be accurately measured by delayed enhancement magnetic resonance imaging (deMRI) with the validation of tetrazolium staining as histological gold standard. **Methods:** Three groups of Sprague-Dawley rats underwent 30 minutes of ischemia by occlusion of the left anterior descending artery. At the end of a designated reperfusion period of 1, 2 or 24 hours, deMRI measurement of infarct size was performed with a spin echo sequence. Corresponding tissue sections from explanted heart were stained with triphenyltetrazolium, and the infarct size was quantitatively compared with deMRI measurements. **Results:** At 2 and 24 hr after infarction, infarct size determined by deMRI was in good agreement with histology, with a difference of $0.53 \pm 3.59\%$ ($n = 5$) and $1.47 \pm 2.19\%$ ($n = 7$), respectively, of the left ventricular cross section area. However, with 1 hr reperfusion, the area of delayed hyper-enhancement overestimated by $7.58 \pm 3.73\%$ ($n = 8$) compared to tetrazolium staining. In addition, infarct size measured at early points of time (1 and 2 hr) was significantly smaller than at 24 hours. **Conclusion:** With tetrazolium staining as a reference, deMRI provides accurate infarct size measurement at a time point as early as 2 hrs after reperfused acute infarction. The data will guide the standardization of deMRI protocols for experimental animal studies and have implications for potential clinical applications.

INTRODUCTION

Delayed enhancement magnetic resonance imaging (deMRI) has been established as a non-invasive, high-resolution imaging technique to assess myocardial infarct size (1). The technique involves an intravenous bolus injection of a low molecular weight, extracellular gadolinium-based contrast agent, and acquiring T_1 weighted post-contrast images with a delay of 10 to 30 minutes

after injection. The contrast enhancement in deMRI is thought to be a result of an increased local distribution of the contrast agent in necrotic tissues, which appear conspicuous against viable myocardium in T_1 weighted images. In clinical applications, deMRI has been shown to have good sensitivity and specificity, and the enhanced regions are correlated with low functional recovery and poor prognosis in patients (2–6).

The diagnostic value of deMRI has been validated by the direct comparison between post-contrast MR images with histological gold standard, where tissue sections are stained with tetrazolium compounds, such as triphenyltetrazolium chloride (TTC) and p-nitroblue tetrazolium (pNBT) (7). The tetrazolium salts dissolve in physiological buffers as colorless solutions. In viable cells, these compounds are reduced by endogenous dehydrogenases and form formazan pigment precipitate as a sign of active metabolism (8). On the other hand, in necrotic tissues, where dehydrogenase activities are depleted, tetrazolium staining has a pale white appearance. In the histological analysis of myocardial specimens, the regions that are negative in

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tetrazolium staining are considered as infarct areas (8). Essentially, infarct size measurement with tetrazolium staining critically depends on the absence of dehydrogenase activities.

The accuracy of deMRI in the very acute phase (within 1 to 2 hours) of myocardial infarction (MI) is important to the characterization of evolving infarct. The applications of deMRI have been reported as early as 60 minutes post infarction in animal models and human subjects, and the myocardium with delayed hyper-enhancement was consistent with the injured coronary territories (9, 10). This outcome demonstrated the sensitivity of deMRI toward irreversible myocardial injury, and the potential benefit to the diagnosis of equivocal patients with acute coronary syndromes. However, to the best of our knowledge, no systematic study has been conducted to specifically look at deMRI measurement of infarct areas with validation by tetrazolium staining as a gold standard. In addition, few data are available to determine the earliest possible time for deMRI to accurately assess the true infarct area, either in human or animal models. To address this knowledge gap, the aims of the current study are two folds: First, we intend to characterize deMRI at 1 and 2 hours of reperfusion in a rat model. Second, we will validate the earliest time point for reliable deMRI measurement in conjunction with tetrazolium staining.

MATERIALS AND METHODS

Animal model

This study conformed to the institutional and national Guide for the Care and Use of Laboratory Animals. A total of 30 10-week old male Sprague-Dawley rats (Harlan, IN) with body weight between 300 to 390 g were used in this study. The rats were anesthetized with sodium pentobarbital (50 mg/kg) intraperitoneally, and respiration was maintained using a rodent ventilator. Real time electrocardiogram (ECG) was monitored throughout the surgery and imaging times.

A thoracotomy was performed, and the chest was opened at the fourth intercostal space to expose the heart. The pericardium was opened with forceps, and a 6.0 suture was passed underneath the left anterior descending coronary artery at the level between the pulmonary trunk and the left atrium. Coronary occlusion was achieved by tightening the suture over a small roll of tissue. The success of occlusion was confirmed by the pale appearance in the area at risk and the immediate changes in ECG profiles, including a significant increase in the amplitude of the QRS complex and elevation of ST segment. In this model, ventricular tachycardia generally occurred at 7 to 15 minutes of ischemia and the Q-wave depressed gradually throughout the whole procedure. The rats were randomly assigned to one of 3 study groups, where all occlusions were maintained for 30 minutes, followed by 1 hour, 2 hours, or 24 hours of reperfusion. Reperfusion was achieved by cutting the knot and releasing the suture from the occlusion. The chest cavity was subsequently closed. Intravenous cannulation was performed in the femoral vein before MR imaging for the injection of gadolinium-diethylenetriamine pentaacetic acid (Gd-DTPA) at a dosage of 0.1 mM/kg. Rats

were kept under deep anesthesia throughout the operation and imaging time.

deMRI protocol

As it has been validated that 24 hours of reperfusion in the rat model results in reliable TTC staining, which fully reflects the true infarct areas, the deMRI protocol was optimized using rats with 30 minutes of ischemia and 24 hours of reperfusion. Each rat was anesthetized with sodium pentobarbital (50 mg/kg), intubated via the trachea, and the respiration was maintained with a rodent ventilator throughout the imaging time. The body temperature was kept constant using a heating pad at 37°C.

All MR images were obtained on a Bruker BIOSPEC 3T/60 system (Bruker Medizintechnik GMBH, Karlsruhe, Germany), using a home-made cylindrical local gradient coil, which, at 100 A, produced gradient fields of 21.30 Gauss/cm, 20.83 Gauss/cm, and 41.20 Gauss/cm in the X, Y, and Z directions, respectively. A custom-made 4.5 cm i.d. birdcage coil was used for excitation and reception. As the purpose of this study was to quantitatively compare the infarct area assessed by MRI and tetrazolium staining in post-mortem tissue sections, we selected a multi-slice spin echo sequence to optimize anatomic information, while maintaining good contrast-to-background ratios. ECG gated short-axis spin echo images (TE = 15.2 ms, TR = 320 ms, FOV = 5 cm, Matrix = 192 × 192, slice thickness = 2 mm, number of slices = 3 or 5 with no interslice spacing) were acquired before and every 5 minutes after Gd-DTPA injection (0.1 mmol/kg) for 35 minutes. A single line of *k*-space was acquired for a given section at every other cardiac cycle. Typically, with a heart rate of 350 bpm and 4 averages, it took 4.5 minute to acquire one set of multi-slice post contrast images.

Rats were euthanized by an intravenous injection of an overdose of pentobarbital immediately after MRI data acquisition. Hearts were excised and infused with 15 mL of 0.5% TTC via the aorta at 37°C, within 3 minutes after the completion of MRI acquisition. The TTC stained heart was fixed with 4% formaldehyde for 24 hours and was then sectioned transversely into 2-mm-thick short-axis slices for alignment with the MR images. Digitized MR and histology images were compared using the procedure described below. The forth post-contrast image, acquired at 15 minutes after Gd-DTPA injection, best correlated with TTC staining in terms of infarct location, shape, and size as a percentage of the left ventricle.

For deMRI at 1 and 2 hours of reperfusion, the chest of the rat was closed immediately after 30 minutes of ischemia. The rat was transferred into the MR scanner, while its respiration was maintained with a rodent ventilator. After localizer images were acquired, Gd-DTPA was injected via the femoral vein cannula at 40 or 100 minutes of reperfusion. Four post-contrast acquisitions were performed at 5 minutes each, and the entire MRI data collection was completed at 60 or 120 minutes of reperfusion. Histological analysis was followed immediately.

Data analysis

Reconstructed MR images and digital photographs of TTC stained corresponding tissue slices were converted to TIFF

format and imported into AIS 6.0 software (Imaging Research Inc., ON, Canada) for analysis. For MR images, only one section from each animal with excellent anatomical appearance, including clearly discernable endo- and epicardial outlines without significant motion artifact or distortion due to irregular cardiac kinetics, was included for data analysis. For deMRI images, the infarct size was determined by quantitative measurements of left ventricular cross section and infarct areas by 3 independent observers. The endocardial and epicardial boundaries of each image were carefully traced by hand. The size of the ventricular cross section and delayed hyper-enhanced region were determined in terms of the number of pixels by drawing regions of interest (ROI) on digitized images. For histology sections, both sides of the TTC stained slice were imaged, and infarct size was calculated as the total TTC-negative area divided by the total area of ventricular cross section from the top and bottom surface. We avoided using automated image analysis software due to the following complications:

1. In the MR delayed enhancement images, the no flow regions at the necrotic centers could appear as non-enhanced.
2. Signal to noise ratios and contrast enhancement could be affected by the heart rate of the subject, thus making it impractical to have a universal threshold for signal intensity cut-off.
3. Local hemorrhage at the infarct regions was stained black by TTC, which was easily distinguished by human observers but consistently mistaken as TTC-positive viable areas by computerized planimetry.

The infarct area as a percentage of the left ventricle from each MR image was compared with that of corresponding TTC-stained section. After a mortality rate of approximately 20% and exclusion of data with ill-defined epi- and endocardial boundaries or motion artifacts, a total of 20 sections with corresponding histology were included for data analysis. Each individual slice was considered as a single data point, and the number of points for each reperfusion period was $n = 8$, $n = 5$, and $n = 7$ for 1, 2, and 24 hours, respectively. Results were expressed as mean $\pm$ standard deviation, with median values, 25th and 75th range, and minimal and maximal values for each group. The differences in infarct size between deMRI and TTC, as well as between time points, were assessed using paired t test, where a p value less than 0.05 is considered as significant.

RESULTS

Without nulling the viable myocardium with inversion-recovery, the epi- and endocardial boundaries were well defined in the post-contrast deMRI images, while contrast enhancement was generally between 20 to 100% above remote viable regions. Although the contrast-to-background ratios were lower compared with inversion recovery methods (13, 14), the distinction between non-enhanced viable myocardium and the delayed enhanced areas was sufficiently clear, with inter-observer variability of less than 3%.

The MR imaging protocol for delayed enhancement was established and validated with myocardial infarction that was followed by 24 hours of reperfusion. Ischemic challenge of 30 minutes was sufficient to cause extensive myocardial infarction in the left descending coronary territory. Large infarct area was consistently resulted in the anterior side of the mid ventricular wall and extending to the lateral and a portion of the posterior wall in the inferior sections toward the apex. The layout of the infarct region was detected with multi-slice T_1 weighted MR sequence, and confirmed by TTC staining. A representative post-contrast MR image acquired with 15 minutes delay and the corresponding TTC stained tissue section are shown in Fig. 1A. With 24 hours of reperfusion, the deMRI at a delay of 15 minutes after Gd-DTPA injection gave the most accurate infarct size measurement compared with TTC. The median infarct size values determined by deMRI and TTC were 39.50 and 39.90% of the left ventricular cross section (Fig. 2), while the means were

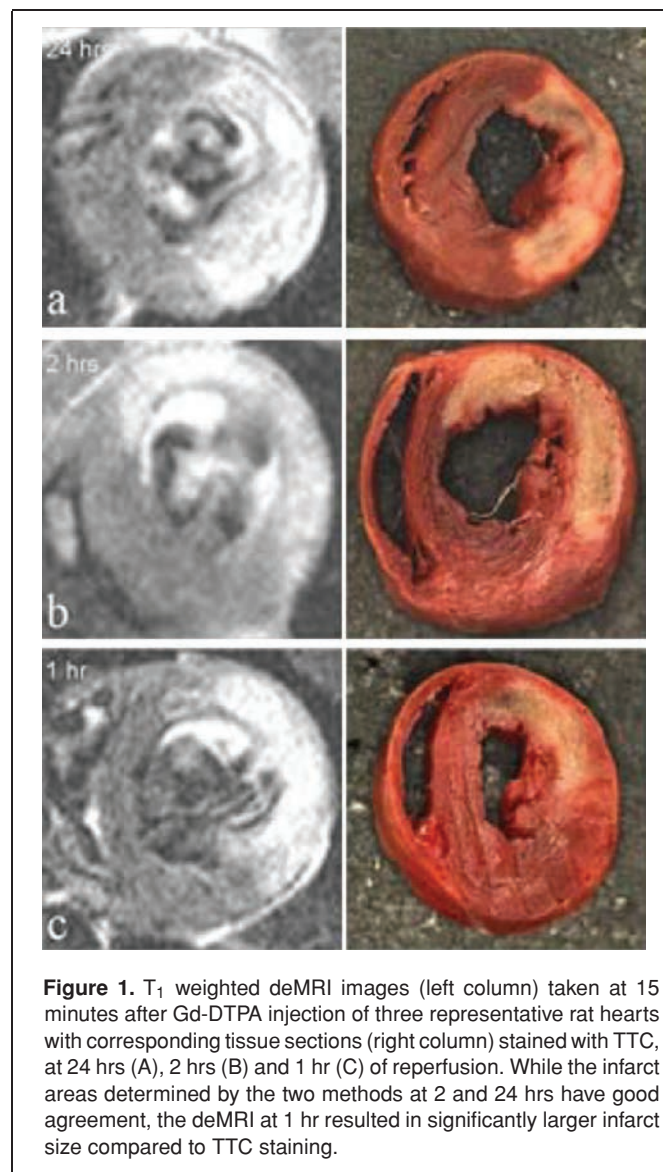


Figure 1. T_1 weighted deMRI images (left column) taken at 15 minutes after Gd-DTPA injection of three representative rat hearts with corresponding tissue sections (right column) stained with TTC, at 24 hrs (A), 2 hrs (B) and 1 hr (C) of reperfusion. While the infarct areas determined by the two methods at 2 and 24 hrs have good agreement, the deMRI at 1 hr resulted in significantly larger infarct size compared to TTC staining.

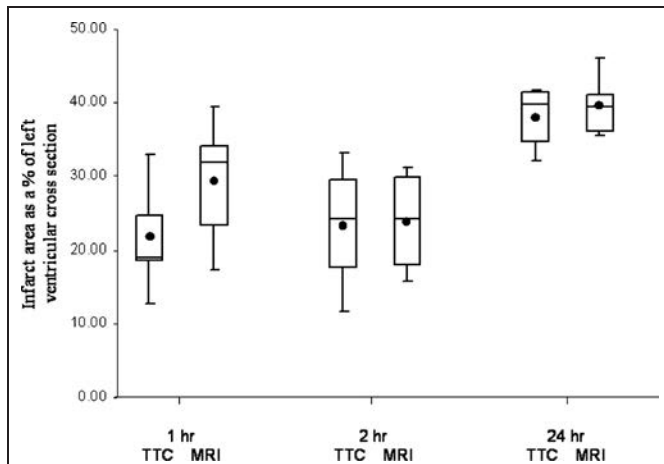


Figure 2. Box plot of infarct size as a percentage of the left ventricular cross section area, measured by TTC and deMRI, at 1 (n = 8), 2 (n = 5) and 24 hours (n = 7) after reperfusion. The horizontal bar indicates the median value; the top and bottom lines are 25th and 75th percentile; the vertical line indicates the range, with minimal and maximal values; the solid dot represents the mean value.

$39.72 \pm 4.25\%$ and $37.96 \pm 4.36\%$, respectively. The mean difference in infarct size measured by the two methods, calculated from each pair of corresponding MR image and tissue section, was $1.47 \pm 2.19\%$ (n = 7) of the left ventricular cross section (Fig. 3). According to paired *t*-test, there was no significant difference in infarct size determined between the two methods ($p = 0.163$).

With 2 hours of reperfusion, there appeared to be a good agreement in the infarct area determined by deMRI and histology. Fig. 1B illustrates typical post contrast deMRI image and the matching TTC stained section. The median infarct size val-

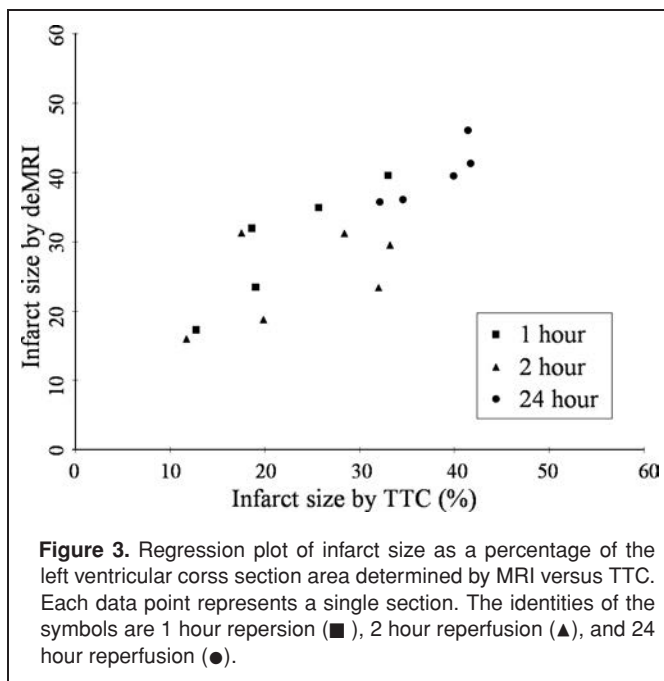


Figure 3. Regression plot of infarct size as a percentage of the left ventricular cross section area determined by MRI versus TTC. Each data point represents a single section. The identities of the symbols are 1 hour reperfusion (■), 2 hour reperfusion (▲), and 24 hour reperfusion (●).

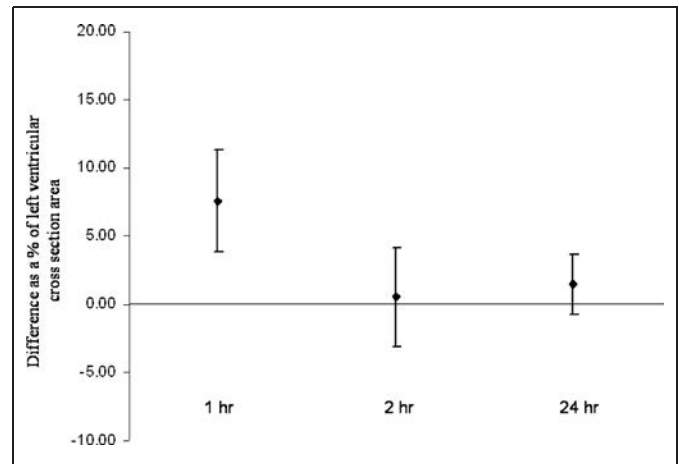


Figure 4. Plot of the differences in infarct area determined from deMRI and TTC staining, in terms of percentage of left ventricular cross section area at 1 (n = 8), 2 (n = 5) and 24 hours (n = 7). The mean is expressed as solid dot, with standard deviations.

ues by deMRI and TTC were identical, at 24.10% of the left ventricular cross section, and the means were $23.80 \pm 7.62\%$ and $23.23 \pm 9.50\%$, respectively (Fig. 2). A quantitative comparison is shown in Fig. 3, where it demonstrates that the difference in infarct size determined by deMRI and TTC staining at a mean of $0.53 \pm 3.59\%$ (n = 5) was insignificant ($p = 0.721$). However, compared to the 24 hour measurements, the infarct size at 2 hours is significantly smaller, with $p = 0.0021$ for deMRI and $p = 0.0093$ for TTC (Fig. 2).

A shorter reperfusion time of 1 hour resulted in the largest discrepancy and deviations in infarct area measured between the two methods (Fig. 4). As demonstrated in Fig. 1c, the boundary between viable and TTC deficient regions were less well defined compared to 2 and 24 hour samples. Small but visible uncharacteristic pink areas were sometimes observed at the peripheral region of the infarct. The median values determined by deMRI and TTC were 31.90 and 19.00%, respectively; the average infarct area by the two methods were $29.38 \pm 8.93\%$ and $21.80 \pm 7.77\%$ (Fig. 2). As shown in Fig. 3, the difference in infarct area determined by deMRI and histology was $7.58 \pm 3.73\%$ (n = 8), which was statistically significant with *p* value of 0.0105. There was no significant difference in infarct size determined by TTC at 1 and 2 hours ($p = 0.705$). Compared with 24 hr, the infarct size at 1 hr was significantly smaller with $p = 0.0475$ for deMRI and $p = 0.0037$ for TTC.

DISCUSSIONS

The current study explores the potential diagnostic value of deMRI for evolving, rather than established, myocardial infarction. Two significant points were demonstrated. First, within 1 hour reperfusion after a 30 min ischemia, the infarct area determined by deMRI was consistently and significantly larger compared to that measured with TTC staining. After longer reperfusion periods, at 2 and 24 hour, deMRI measurements become more consistent with histology. The discrepancy in

infarct size measured by the two methods at 1 hour reperfusion appears to reflect the nature of different surrogate markers for irreversible tissue damage. Second, there was significant increase in infarct size during a 24 hour reperfusion period. In this respect, measurements with both deMRI and histology were consistent.

Tetrazolium staining essentially detects the absence of dehydrogenase activity as a marker for necrosis. Previous studies have demonstrated that it takes a minimal period of time for dehydrogenase activity to be completely depleted in necrotic cells, accompanied by proteolytic degradation and washout of intracellular components (11, 12). In a rat model of acute infarct with 90 minute ischemia, at least 1 hour of time is needed to elapse before the full extent of infarct is faithfully captured by TTC staining (12). For human myocardium, this minimum time is unknown, and indirect evidence has been derived with *ex vivo* studies (11). If performed too soon after infarction, tetrazolium staining may result in false color development in apparently non-viable myocardium. In the current study, it is possible that this scenario has contributed to errors in infarct measurement by TTC staining at 1 hour reperfusion, as evident by the poorly defined boundary between viable and TTC-negative areas, as well as the presence of uncharacteristic pink regions. In contrast to the previous report, where 1 hour of reperfusion was sufficiently long for reliable TTC staining, these observations are indicative of residual endogenous dehydrogenase activities in the possibly non-viable myocardium at 1 hour reperfusion, suggesting that in this model it would require reperfusion periods longer than 1 hour. The false color development in non-viable myocardium could have resulted in both relative underestimation of the true infarct areas and elevated standard deviations.

On the other hand, deMRI detects changes in interstitial volume, using non-specific, small molecule extracellular contrast agents, such as Gd-DTPA (13). After intravenous injection, these agents rapidly extravasate from the blood vasculature and diffuse into the interstitium. By targeting changes in extracellular space, deMRI takes the advantage of severely compromised sarcolemmal and plasma membrane systems and reflects differential pharmacokinetics of highly diffusible contrast agents. The nondiscriminatory nature of non-specific contrast agents may be susceptible to interstitial volume changes other than necrosis. For instance, it has been shown that edema is frequently associated with evolving myocardial infarct, and that it is resolved over time while the necrotic regions become stabilized (9, 15). Enhancement in T_2 weighted gradient echo images as a result of edema is sometimes associated with the peripheral regions of infarct (15, 16). Given its relatively transient nature, the presence of edema has been proposed as an criterion for the distinction between new and existing infarct (9). Unfortunately, there is no well-validated gold standard to quantify edema and to correlate with deMRI and histology. While edema is associated with increased interstitial space, it is thus likely to result in an overestimation of infarct size by deMRI. In addition, larger infarct size could also be due to swelling without additional myocyte loss. However, our data could not confirm or rule out this possibility.

Both incomplete enzyme depletion and reversible changes in interstitial volume are transient, dynamic processes associated

with the very early stage of acute myocardial injuries and will resolve over time. The potential contributions from these factors to the errors in early infarct size measurements are consistent with the observation that with 2 hours or longer reperfusion, infarct measurement with deMRI is in good agreement with histology.

The overall increase in infarct size by 24 hours indicate that although both deMRI and TTC were able to capture the true infarct size at 2 hours after reperfusion, the eventual extent of irreversible damage could be significantly larger. This could be explained by infarction being an dynamically evolving process, where myocardium at the peripheral regions of the infarct continues to lose viability during reperfusion. Evidence has shown that reperfusion and reoxygenation can induce high levels of apoptosis by oxidative stress, and that between 5 to 25% of the myocytes within the area at risk could become apoptotic during the 15 hours after infarction. While apoptosis is an energy-dependent event taking place without the rupture of the plasma membrane, apoptotic cells are not detected by either TTC or necrosis-avid imaging agents (17). Thus, early infarct measurement within a few hours after reperfusion could result in an underestimation of the extent of true irreversible injury.

Animal models of myocardial ischemia and reperfusion, including rodents, are widely used in cardiovascular research, but no existing literature has systematically addressed the timing of deMR imaging and tetrazolium staining protocols early after infarction. Based on the observations from the current study, the following recommendations may be made:

1. deMRI is sensitive enough to detect the presence of evolving myocardial infarction as early as 1 hour after reperfusion. However, the accuracy of infarct size measurement at this time point is prone to complications from reversible interstitial volume changes associated with infarction, and from the incomplete depletion of dehydrogenase activities which can lead to false histological measurements by tetrazolium staining.
2. With 30 minutes of ischemia in rats, deMRI and tetrazolium determination of infarct size have good agreement if the measurements are performed after 2 hours of reperfusion.
3. The extent of irreversible damage as a consequence of ischemia/reperfusion can be significantly larger than the apparent infarct size measured at 1 or 2 hours of reperfusion.

The current results suggest that the use of small molecule, extracellular contrast agents can be very sensitive to changes in interstitial space in the very early phase of myocardial necrosis. These data may serve as a proof of principle that deMRI could potentially be used to detect the very early stage of irreversible myocardial damage. As an early and non-invasive surrogate marker, deMRI may serve as a valuable reference for assessing the outcome of therapeutic interventions. Clinically, treatments such as angiotensin-converting enzyme inhibitor, beta-blockers and aspirin have shown cardioprotective

effects as reflected from long-term survival and cardiac functions (18–20). An early imaging end point for acute infarct characterization would facilitate patient stratification and have prognostic predictive values. However, caution must be taken to extrapolate the observations in the current study to clinical diagnostic purposes, given the physiological and structural differences in human and rodent myocardium. In addition, the feasibility of such early application will eventually be determined by the practicality and constraints from the operational aspects of MRI, such as patient monitoring and safety while inside the magnet.

While the current data demonstrate the correlation between contrast enhancement by deMRI and TTC staining at an early stage in reperfused acute infarct, the utility of deMRI in detecting non-reperfused infarct in the hyperacute stage will require further validations. To the best of our knowledge, currently there is no histological gold standard for the assessment of non-reperfused infarction at the very early stage (within 1 to 2 hours post occlusion). In rats, 6 hours of permanent ligation of the left coronary artery is necessary for reliable tetrazolium staining, as opposed to 1 hour with reperfused infarction (12, 21). As the collateral flow of rat myocardium is rather low, permanent occlusion of the coronary artery will result in no flow to the coronary territory (22). In the absence of perfusion, while dehydrogenases are not effectively washed out from local myocardium, TTC staining will be false-positive as viable (21). This delay renders the study of early non-reperfused MI with deMRI unauthenticated. However, it is worth mentioning that in clinical settings, hypoperfused myocardium is identifiable with first-pass Gd-DTPA enhancement, although such measurements may not directly reflect the extent of infarction early after occlusion (23).

The limitations of the current study include:

1. At 4.5 minute per set of deMRI images, the gain in spatial resolution was at the expense of temporal resolution. More post-contrast data points would have led to further optimization of imaging protocols.
2. As uncertainties in TTC staining could be at least partially responsible for the relative overestimation of infarct area by deMRI at 1 hr after reperfusion, it precludes studies earlier than 1 hr, whereas the deMRI is technically feasible. However, besides tetrazolium staining, there lacks an alternative methodology as a second gold standard that independently validate the true infarct size measurements.

In conclusion, the current data indicate that deMRI is sensitive not only to established necrotic myocardium but also to evolving infarct. To achieve accurate infarct size measurement, a defined period of time is needed before a surrogate marker, such as changes in interstitial space and depletion of endogenous enzyme, can faithfully reflect the true necrotic region.

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