Chronic Manifestation of Postreperfusion Intramyocardial Hemorrhage as Regional Iron Deposition
A Cardiovascular Magnetic Resonance Study With Ex Vivo Validation

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Background—Intramyocardial hemorrhage frequently accompanies large reperfused myocardial infarctions. However, its influence on the makeup and the ensuing effect on the infarcted tissue during the chronic phase remain unexplored.

Methods and Results—Patients (n=15; 3 women), recruited after successful percutaneous coronary intervention for first segment–elevation myocardial infarction, underwent cardiovascular magnetic resonance imaging on day 3 and month 6 after percutaneous coronary intervention. Patients with hemorrhagic (Hemo+) infarctions, as determined by T2* cardiovascular magnetic resonance on day 3 (n=11), showed persistent T2* losses colocalized with scar tissue on the follow-up scans, suggesting chronic iron deposition. T2* values of Hemo+ territories were significantly higher than nonhemorrhagic (Hemo−) and remote territories (P<0.001); however, T2* values of nonhemorrhagic (Hemo−) and remote territories were not different (P=0.51). Canines (n=20) subjected to ischemia-reperfusion injury (n=14) underwent cardiovascular magnetic resonance on days 3 and 56 after ischemia-reperfusion injury. Similarly, sham-operated animals (Shams; n=3) were imaged using cardiovascular magnetic resonance at similar time points. Subsequently, hearts were explanted and imaged ex vivo, and samples of Hemo+, Hemo−, remote, and Sham myocardium were isolated and stained. The extent of iron deposition ([Fe]) within each sample was measured using mass spectrometry. Hemo+ infarcts showed significant T2* losses compared with the other (control) groups (P<0.001), and Perls stain confirmed localized iron deposition. Mean [Fe] of Hemo+ was nearly an order of magnitude greater than that of the control groups (P<0.001), but no significant differences were observed among the control groups. A strong linear relationship was observed between log(T2*) and −log([Fe]); R²=0.7 and P<0.001. The monoclonal antibody Mac387 stains, along with Perls stains, showed preferential localization of newly recruited macrophages at the site of chronic iron deposition.

Conclusions—Hemorrhagic myocardial infarction can lead to iron deposits within the infarction zones, which can be a source of prolonged inflammatory burden in the chronic phase of myocardial infarction. (Circ Cardiovasc Imaging. 2013;6:218-228.)

Key Words: acute myocardial infarction • cardiac MRI • hemorrhage • inflammation • ventricular remodeling

Specific pathological conditions can mediate increased tissue levels of iron in a number of organs, including the brain, liver, and heart. In these settings, biodegradation of heme, harbored by red blood cells (RBCs), is considered to be the most significant source of iron. In the heart, chronic deposition of iron has been known to occur predominantly in hemochromatosis, in blood transfusion therapy for thalassemia major, and in sickle-cell anemia. Abnormal accumulation of iron in the heart has been associated with poor cardiac function and conduction abnormalities.

Clinical Perspective on p 228

Pathological pooling of RBCs within the heart muscle is known to take place in the setting of reperfused myocardial infarction. Although the endothelial cells have a higher
tolerance for ischemia than cardiomyocytes, prolonged periods (>90 minutes) of no-flow ischemia in myocardial tissue lead to oncosis (cell death) of the vascular endothelium and thus to a breakdown of the microvascular barrier. Therefore, a delayed reestablishment of epicardial coronary blood flow after acute coronary occlusion may lead to the extravasation of erythrocytes (hemorrhage) with severe consequences for regional perfusion. In fact, gross bleeding within the heart muscle is a frequently encountered hallmark of large myocardial infarctions.22–25 The observed correlate with respect to blood flow is known as no-reflow.26,27

Although the prognostic significance of large infarctions with microvascular obstructions (MVOs) has been well studied,28–30 interest in the clinical consequences of intramyocardial hemorrhage has been increasing only recently. Hemorrhagic infarctions have been shown to be associated with adverse left ventricular (LV) remodeling,21–34 late arhythogenic risk,35 and major adverse cardiovascular events.36 However, the long-term fate of acute repurposed myocardial hemorrhage and its effects on the heart remain largely unexplored. In this study, we investigated whether (1) acute repurposed hemorrhage specifically imparts compositional changes in chronically infarcted myocardial tissue and (2) if so, whether such compositional differences mediate prolonged inflammation in the chronic phase of infarction. In particular, we hypothesized that acute repurposed myocardial hemorrhage leads to chronic iron deposition localized within the infarcted territories in the chronic phase. We tested this hypothesis using cardiovascular magnetic resonance (CMR) imaging in a pilot patient population who underwent successful coronary revascularization for first ST-segment–elevation myocardial infarction. Furthermore, using a proof-of-concept canine model subjected to ischemia–repurposed (I/R) injury, we validated our findings in humans. Subsequently, we tested the hypothesis whether repurposed-induced chronic iron deposition leads to a prolonged inflammatory activity in the chronic phase of infarction. We tested this hypothesis by histopathologic analysis of chronically infarcted canine myocardium with a history of acute repurposed hemorrhage.

Methods

Patient Selection and CMR Imaging Studies

Patients were enrolled after provided informed consent and study according to the protocols approved by the Institutional Review Board at the Foothills Medical Center (Calgary, AB, Canada). Patients with acute ST-segment–elevation myocardial infarction (n=15; 3 women; mean age=58±8 years) meeting the American Heart Association diagnostic criteria were enrolled in the study, only if successful percutaneous coronary intervention (PCI) (Thrombolysis in Myocardial Infarction flow grade 3 post-PCI) was performed within 12 hours of the onset of symptoms. Patients were excluded from the study if they had previous myocardial infarctions or had contraindications for a CMR study. All enrolled patients underwent CMR 3 days after PCI and again at 6 months after initial enrollment in a clinical 1.5-T MRI system (MAGNETOM Avanto, Siemens Medical Solutions, Erlangen, Germany).

Cine steady-state free precession37 (repetition time [TR]/echo time [TE]=3.32/1.16 milliseconds, flip angle=65°, readout bandwidth (BW)=930 Hz/pixel, 25 cardiac phases, in-plane resolution=1.8x1.8 mm²), T2*–weighted18,39 (TR=240 milliseconds; 6 TEs=2.6 milliseconds with ΔTE=2.2 milliseconds, flip angle=10°; BW=355 Hz/pixel; in-plane resolution=1.6x1.6 mm²), and late gadolinium enhancement40 (LGE; inversion-recovery prepared fast low-angle shot; TR =1 R-R interval; TEs=3.32 milliseconds; BW=235 Hz/pixel, 1.6x1.6 mm²) images of contiguous short-axis sections of the LV along with 2-, 3-, and 4-chamber long-axis views of the heart were acquired. Commonly used imaging parameters were slice thickness of 10 mm and number of averages of 1. Note that our TE selection for the T2*-weighted acquisitions was determined with the goal of optimizing image quality and T2* sensitivity. Although a slice thickness of 6 to 8 mm and TR of 2 R-R intervals are used as standard for LGE images, we chose to acquire thicker slices at a TR of 1 R-R interval primarily to reduce the breath-hold time for the patients. The heart rates of the patients in our study were sufficiently low that excellent contrast between the remote and infarcted myocardium could still be achieved.

Proof-of-Concept Studies in Canines

Animal Preparation and CMR Studies

Canines (n=23; 20–25 kg) were studied according to the protocols approved by the Institutional Animal Care and Use Committee of Northwestern University (Chicago, IL). Animals were subjected to I/R injury (Infarct group, n=20) by fully occluding the left anterior descending artery (Figure 1 in the online-only Data Supplement) for 3 hours followed by repurposition. CMR studies were performed on a clinical 1.5-T MRI system (MAGNETOM Avanto, Siemens Medical Solutions, Erlangen, Germany) on days 3 (acute) and 56 (chronic) after I/R injury. Sham-operated animals (Sham group; n=3) underwent CMR at similar time points. Cine steady-state free precession (TR/TE=3.5/1.75 milliseconds; flip angle=70°; 25–30 cardiac phases and BW=930 Hz/pixel, T2*–weighted (TR=240 milliseconds; 6 TEs=18.4 milliseconds with ΔTE=3.0 milliseconds; flip angle=12° and BW=566 Hz/pixel), and LGE images (inversion-recovery prepared steady-state free precession; TR/TE=3.5/1.75 milliseconds; flip angle=40° and BW=1002 Hz/pixel) of contiguous short-axis sections covering the entire LV and the 3 long-axis views were acquired at mid-diastole. As in patient studies, the TE selection for the T2*-weighted acquisitions was determined with the goal of optimizing image quality and T2* sensitivity. Commonly used imaging parameters were in-plane resolution of 1.5x1.3 mm², slice thickness of 8 mm, and number of averages of 1. On day 56, animals were euthanized, and their hearts were excised and cut into roughly 1-cm-thick slices. Ex vivo 2D T2+-weighted and LGE images of each slice were acquired using the in vivo imaging parameters.

Isolation of Tissue Samples and Histopathology

The freshly excised heart from each animal was sliced and stained with triphenyl tetrazolium chloride41 (TTC) to histochemically validate irreversible myocardial damage and to delineate the infarcted territories from viable myocardium. Remote, hemorrhagic (Hemo+), and nonhemorrhagic (Hemo–) myocardium were identified on the basis of ex vivo CMR and TTC staining as explained in the Image Analysis section. Hemo+ and Hemo– infarcts were cut out into 0.5- to 0.8-cm² samples from densely infarcted areas. Similarly, from each slice, at least 2 samples of TTC-stained viable myocardium were isolated (remote). From each sham slice, at least 2 samples of TTC-stained normal myocardium were isolated. Contiguous 5-μm sections were obtained from a representative sample of each of the Hemo+, Hemo–, remote, and sham groups from every animal. These sections were stained with hematoxylin and eosin stain, Masson Trichrome stain for fibrosis, Perls stain for iron, and monoclonal antibody Mac387 stain (Dako, Carpinteria, CA) for newly recruited macrophages, and sections were imaged at ×100 and ×40 magnifications.

Inductively Coupled Plasma Mass Spectrometry

The amount of iron deposited within each myocardial sample ([Fe]sample in milligrams per gram of tissue) was measured using a quadrupole-based X Series 2 ICP-MS (Thermo-Fisher Scientific) equipped with Collison Cell Technology to reduce interference from doublets. Whole-heart Fe content ([Fe]heart) was calculated by weight-averaging the Fe content of all the constituent samples. In all, 55
Image analysis

Image analyses were performed using cmr42 (Circle Cardiovascular Imaging Inc, Canada; Figure II in the online-only Data Supplement). T2* maps were constructed by fitting the multiecho T2*-weighted images to a monoexponential decay (Figure III in the online-only Data Supplement). For the in vivo CMR images from both patients and animals, remote myocardium was defined as the region showing no hyperintensity on LGE images. A reference region of interest was drawn in remote myocardium on both LGE and T2*-weighted images. Infarcted myocardium was defined as the hypertense region on LGE images with mean signal intensity at least 5 SDs above that of the reference region of interest.35 On the acute phase LGE images, hypointense regions of MVO were manually included as part of the infarcted myocardium. Hemo+ myocardium was defined as infarcted myocardium with hypointense signal on the T2*-weighted image acquired at the last echo (TE=13.7 milliseconds for patients and 18.4 milliseconds for animals) with mean signal intensity of at least 2 SDs below that of the reference region of interest.39,41 The last echo was used to ensure adequate T2* weighting. The different thresholds used for the detection of infarcted territories and hemorrhagic zones have been previously described by others39,42,43 and are a reflection of the sensitivity of the T1 and T2* approaches used for identifying the respective markers (Gd for T1 and byproducts of heme for T2*).

Hemo− myocardium was defined as the region positive for infarction on LGE images but negative for hemorrhage on the corresponding T2*-weighted images. For the in vivo T2*-weighted images, care was taken not to include infarcted territories and hemorrhagic zones that would have been previously described by others39,42,43 and are a reflection of the sensitivity of the T1 and T2* approaches used for identifying the respective markers (Gd for T1 and byproducts of heme for T2*).

For patients, mixed-model linear regression with Bonferroni corrections was performed to determine whether %Infarct and %Hemo were predictors of LV remodeling parameters (EDV, EF, ESV, and EDSI) in the chronic phase at 6 months post-PCI.

For animals, in vivo T2* slice, ex vivo T2* slice, and [Fe]total were compared among the Hemo+, Hemo−, remote, and sham groups using mixed-model linear regression with Bonferroni corrections (P<0.0001). We tested the null hypothesis that there was no difference among the 4 different groups for each parameter of interest. Canines were entered as random effects, whereas samples from each heart (or myocardial slice) were nested in the analysis to account for repeated measurements from a single heart (or myocardial slice). Paired t test was used to compare in vivo T2* heart values obtained from CMR studies on days 3 and 56. The relationship between in vivo T2* heart (from days 3 and 56 CMR studies) and the corresponding ex vivo T2* heart was evaluated. Similarly, the relationship between log(ex vivo T2* heart) and −log([Fe]total) was evaluated.

LV mass, EDV, ESV, EF, EDSI, %Infarct, and %Hemo were compared in canines between acute (day 3) and chronic (day 56) phases postperfusion using paired t test. Simple and multivariable regression analyses were performed to determine whether %Infarct and %Hemo were predictors of LV remodeling (EDV, ESV, EF, and EDSI) at in the chronic phase (day 56) postperfusion.

Results

Chronic Iron Deposition in Humans After Hemorrhagic Myocardial Infarction

In the acute phase (day 3), 11 patients were identified positive for hemorrhagic myocardial infarction (Table 1; regions: 5 lateral, 3 anteroseptal, and 3 inferior lateral), whereas 4 patients had nonhemorrhagic infarctions (2 lateral, 1 anteroseptal, and 1 inferior lateral). A representative set of CMR images acquired on day 3 and 6 months post-PCI from a patient with hemorrhagic infarction is shown in Figure 1A. Relative to the remote myocardium, significant T2* decreases were observed in the acute infarct territories in 11 patients and remained reduced on the 6-month follow-up images. On day 3 CMR, all the imaging slices that showed significant T2* losses within the infarcted territories also showed MVO on the corresponding LGE images. In the 4 patients negative for hemorrhage on day 3, T2* values of the infarcted zones were similar to the remote territories on the 6-month follow-up scans. Comparison of in vivo T2* slice among the different groups obtained during the acute and chronic phases of infarction showed that T2* of Hemo+ (44 slices from acute phase and 40 slices from chronic phase) was significantly lower than those of the other groups (P<0.0001; Figure 1B), whereas T2* slice values of remote (70 slices from acute phase and 64 slices from chronic phase) and Hemo− (26 slices from acute phase and 24 slices from chronic phase) tissue groups were not different (P=0.51). T2* heart values of remote, Hemo−, and Hemo+ tissues did not differ significantly between acute and chronic phases (remote: P=0.21; Hemo−: P=0.12; Hemo+: P=0.19). On average, we observed a 40% reduction in T2* of Hemo+ regions compared with the control regions, remote, and Hemo−. In vivo T2* heart values from these tissues on day 3 and month 6, regressed against one another, showed a strong correlation (y=0.79x+5.85, where y is T2* heart at month 6, and x is T2* heart on day 3; R²=0.7; P<0.001; Figure 1C).
Morphological and Functional Characteristics of LV in Patients Post-PCI

Compared with the patients with Hemo− infarctions, patients with Hemo+ infarctions had larger LV mass index, larger %Infarct volume, larger EDV, larger ESV, and lower EF at both acute (day 3) and chronic (month 6) phases post-PCI (Table 1; *P*<0.001 for all cases). There was no significant difference between the EDSI of patients with Hemo+ and Hemo− infarctions at 3 days post-PCI (*P*=0.31). However, EDSI of patients with Hemo+ infarctions was significantly higher than that of patients with Hemo− infarctions at 6 months post-PCI (*P*<0.001).

Between the acute and chronic phases, LV mass index and %Infarct volume decreased in patients with both Hemo+ and Hemo− infarctions (*P*<0.001 for all cases). Patients with Hemo− infarctions had no significant difference in EDV between day 3 and month 6 post-PCI (*P*=0.14), but there was a significant decrease in ESV (*P*=0.024) and a significant

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**Figure 1.** Clinical T2* cardiovascular magnetic resonance (CMR) for detecting chronic iron deposition after hemorrhagic myocardial infarction in patients. **A**, Representative CMR images (acquired from a 42-year-old patient after successful percutaneous coronary intervention) with significant T2* loss (arrows) at the site of acute and chronic myocardial infarction (identified by late gadolinium enhancement [LGE] imaging, arrows) are shown. **B**, Mean T2* of Hemo+ sections were significantly lower than those of remote and Hemo− sections (^*P*<0.001) on both acute and chronic CMR studies. However, no difference was observed between T2* measures obtained from remote and Hemo− territories in both acute and chronic states. No significant changes in T2* were observed between acute and chronic phases in remote, Hemo−, and Hemo+ tissues. **C**, Linear regression analysis between T2*heart measurements obtained on day 3 (acute) and month 6 (chronic) showed strong correlations.

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**Table 1. Patient Clinical Features and LV Morphological and Functional Characteristics After PCI**

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Hemorrhagic (n=11)</th>
<th>Nonhemorrhagic (n=4)</th>
<th><em>P</em> Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age,* y</td>
<td>52±11</td>
<td>60±9</td>
<td>0.14</td>
</tr>
<tr>
<td>Male sex, n (%)</td>
<td>9 (81)</td>
<td>3 (75)</td>
<td>0.02</td>
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<td>Diabetes mellitus, n (%)</td>
<td>0 (0)</td>
<td>2 (50)</td>
<td>0.005</td>
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<td>Smoking history,* n (%)</td>
<td>9 (81)</td>
<td>1 (25)</td>
<td>0.19</td>
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<tr>
<td>Hypertension, n (%)</td>
<td>0 (0)</td>
<td>2 (50)</td>
<td>0.005</td>
</tr>
<tr>
<td>Hyperlipidemia, n (%)</td>
<td>0 (0)</td>
<td>1 (25)</td>
<td>0.001</td>
</tr>
<tr>
<td>Time from symptoms to reperfusion,* median (range), h</td>
<td>5.2 (1.9–11.7)</td>
<td>3.8 (2.1–5.3)</td>
<td>0.27</td>
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<tr>
<td>TIMI flow pre-PCI,* n (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Grade 0</td>
<td>5 (46)</td>
<td>2 (50)</td>
<td></td>
</tr>
<tr>
<td>Grade 1</td>
<td>3 (27)</td>
<td>2 (50)</td>
<td>0.87</td>
</tr>
<tr>
<td>Grade 2</td>
<td>3 (27)</td>
<td>0 (0)</td>
<td></td>
</tr>
<tr>
<td>TIMI flow post-PCI, n (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Grade 3</td>
<td>11 (100)</td>
<td>4 (100)</td>
<td>...</td>
</tr>
<tr>
<td>LV end-diastolic volume index, mL/m²</td>
<td>62.7±7.7</td>
<td>74.2±5.4</td>
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<tr>
<td>LV end-systolic volume index, mL/m²</td>
<td>31.9±8.9</td>
<td>40.1±7.8</td>
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<tr>
<td>LV ejection fraction, %</td>
<td>46.2±5.1</td>
<td>40.6±13.1</td>
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<tr>
<td>LV end-diastolic sphericity index</td>
<td>0.44±0.14</td>
<td>0.51±0.17</td>
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<tr>
<td>LV mass index, g/m²</td>
<td>95.5±13.8</td>
<td>86.6±15.6</td>
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<tr>
<td>Infarct volume, %LV</td>
<td>17.6±5.7</td>
<td>12.6±5.5</td>
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<tr>
<td>Hemorrhage volume, %LV</td>
<td>5.0±3.2</td>
<td>2.1±1.6</td>
<td></td>
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</tbody>
</table>

Data are reported as mean±SD, except when noted otherwise. LV indicates left ventricle; PCI, percutaneous coronary intervention; and TIMI, Thrombolysis in Myocardial Infarction.

*Nonsignificant.*
increase in EF ($P=0.013$). In contrast, patients with Hemo+ infarctions ($P=0.14$) had significant increases in EDV and ESV ($P<0.001$ for EDV and $P=0.007$ for ESV) and a significant decrease in EF ($P=0.01$) between the acute and chronic phases. EDSD of patients with Hemo− infarctions was not significantly different between the acute and chronic phases ($P=0.09$), but EDSD of patients with Hemo+ infarctions increased significantly ($P=0.02$). Among the patients with Hemo+ infarctions, %Hemo volume decreased significantly between the acute and chronic phases ($P=0.003$).

Multivariable regression analysis showed that both %Infarct volume and %Hemo volume measured at 3 days post-PCI were significant predictors of change in EDSD between the acute and chronic phases (%Infarct volume: $\beta=1.59, P=0.018$; %Hemo volume: $\beta=2.02, P=0.026$). Simple regression analysis showed that %Hemo volume measured at 6 months post-PCI is a significant predictor of percentage change in EDSD between the acute and chronic phases ($\beta=3.25; P=0.018$). However, a separate multivariable regression analysis showed that only %Infarct volume measured at 6 months was a significant predictor of the change in EDSD between the acute and chronic phases ($\beta=0.87; P=0.015$). However, neither %Infarct volume nor %Hemo volume measured at both 3 days and 6 months post-PCI could predict changes in other LV remodeling parameters (EDV, ESV, and EF).

**Acute Reperfusion Hemorrhage Leads to Chronic Iron Deposition in Canine Hearts**

**Cardiovascular Magnetic Resonance**

Four animals from the infarcted group died within the first 2 hours of establishing reperfusion despite rescue efforts. In 2 animals, reperfusion was not established because of occluder failure. These animals were excluded from the study. The remaining 14 animals from the infarcted group sustained acute hemorrhagic infarctions as observed from the acute phase (day 3) CMR studies. Of these 14 animals, 3 animals were euthanized on day 3 for gross and histopathologic analysis of acute hemorrhagic infarctions. The remaining 11 animals with I/R injury were allowed to recover into a chronic phase and underwent CMR on day 56 postreperfusion. Subsequently, the animals were euthanized, and tissue analysis was performed. The 3 sham animals survived the duration of the study and successfully underwent CMR studies on days 3 and 56, on which the animals were euthanized and their hearts were analyzed. All the data reported from canines in this article (except the histology from acute myocardial infarction) are from the Sham group and animals surviving I/R injury to day 56.

Representative T2* maps and LGE images obtained from the acute phase (day 3), chronic phase (day 56), and ex vivo CMR studies (post euthanization on day 56) are shown in Figure 2. Significant T2* decreases were observable within the infarcted territories in both the acute and chronic phases. Consistent with the patient studies, all the imaging slices on day 3 CMR that showed significant T2* losses within the infarcted territories also showed MVO on the corresponding LGE images. In vivo T2*slice values of Hemo+ territories (33 slices from acute phase and 31 slices from chronic phase) were significantly lower than those of the control groups at acute and chronic phases ($P<0.0001$ for both the cases; Figure 3A). In vivo T2*heart values between Hemo− (23 slices from acute phase and 22 slices from chronic phase), remote (56 slices from acute phase and 53 slices from chronic phase), and sham (20 slices from both acute and chronic phases) groups were not different (remote versus sham: $P=0.72$; remote versus Hemo−: $P=0.81$) during both acute and chronic phases. No significant differences in in vivo T2*heart of Hemo−, remote, and sham territories were observed between acute and chronic phases (Hemo−: $P=0.34$; remote: $P=0.35$; sham: $P=0.26$), but there was a small but significant decrease in in vivo T2*heart of Hemo+ territories in the chronic phase at $P<0.05$ level ($P=0.03$).

Comparison of mean ex vivo T2*slice among the different groups showed that only Hemo+ was significantly different from the control groups ($P<0.0001$; Figure 3B). In vivo T2*heart values from these tissues on days 3 and 56, regressed individually against ex vivo T2*heart estimates, showed very strong correlations ($y=0.52x+16.18, R^2=0.72$; $y=0.56x+14.89, R^2=0.9$, where $y$ is in vivo T2*heart on day 3, $y$ is in vivo T2*heart on day 56, and $x$ is ex vivo T2*heart; $P<0.001$ for both cases; Figure 3C). Similar to patients, on average, a 40% decrease in T2* was observed in regions of hemorrhagic infarctions compared with the control groups at 1.5T.

**Histology**

Representative histopathologic images of acute and chronic myocardial infarctions are shown in Figure 4. Myocardial tissue analysis from animals euthanized on day 3 showed that I/R injury showed large myocardial infarctions evidenced by positive staining in TTC with internal bleeding at the core of the myocardial infarction (Figure 4A). Hematoxylin and eosin stains confirmed the tissue damage and morphological alterations in regions positive for infarction in TTC staining (Figure 4A1). Hematoxylin and eosin stains also showed extravasation of RBCs into the interstitial space of the infarcted regions. Perls stains confirmed a local accumulation of iron within the infarcted areas (Figure 4A3). Hemorrhagic infarctions in the chronic phase on TTC staining showed a visually evident yellowish-brown discoloration extending from the endocardial border to the core of the infarct (Figure 4B). Hematoxylin and eosin stains from the same tissue (Figure 4B1) confirmed the presence of extensive tissue damage, and Masson Trichrome stain (Figure 4B2) showed extensive collagenous tissue in the infarct zone. Perls stain of corresponding myocardial territories showed a persistent and heterogeneous deposition of iron within the infarction (Figure 4B3). Iron was also found to be interspread among viable cells and intact blood vessels (lower inset of Figure 4B3 obtained from infarct border). For a given stain, remote (noninfarcted) tissue sections were similar between acute and chronic phases but were markedly different in infarcted tissue sections (Figure 4A4−A6 and 4B4−4B6). The monoclonal antibody Mac387 staining of chronic infarctions showed that newly recruited macrophages were highly colocalized with the chronic iron deposits identified on Perls stain (Figure 5). Minimal or no macrophages were observed in the infarcted myocardial territories that were devoid of iron deposits. This suggested that there was an active and...
prolonged inflammatory process associated with chronic iron deposition within infarcted territories.

**Mass Spectrometry**

ICP-MS data showed that the mean iron content in Hemo+ samples ([Fe]_{sample}) was significantly elevated compared with the Hemo−, remote, and sham samples (P<0.0001; Figure 6A). However, no significant differences in the mean iron content were observed among sham, remote, and Hemo− groups (remote versus sham: P=0.67; remote versus Hemo−: P=0.31). Regressions between ex vivo T2*_{heart} estimates and tissue iron content ([Fe]_{heart}) also showed a strong correlation (log (ex vivo T2*_{heart})=−0.26log ([Fe]_{heart})+1.21; R²=0.7; P<0.001; Figure 6B).

**Morphological and Functional Characteristics of LV in Canines After I/R Injury**

Consistent with the patient studies, LV mass index, %Infarct volume, and %Hemo volume decreased significantly between acute (day 3) and chronic (day 56) phases postreperfusion (Table 2; P<0.001 for both cases). EDV, ESV, and EDSI increased significantly between the acute and chronic phases (EDV: P=0.034; ESV: P=0.012; EDSI: P=0.008), whereas EF decreased significantly (P=0.021).

Consistent with the patient studies, multivariable regression analysis showed that both %Infarct volume and %Hemo volume measured at 3 days postreperfusion were significant predictors of percentage change in EDSI between acute and chronic phases (%Infarct volume: β=0.81, P=0.014; %Hemo volume: β=1.82, P=0.034). Simple regression analysis showed that %Hemo volume measured at 56 days postreperfusion is a significant predictor of percentage change in EDSI between acute and chronic phases (β=2.82; P=0.031). However, a separate multivariable regression analysis showed that only %Infarct volume measured at 56 days postreperfusion was a significant predictor of percentage change in EDSI between acute and chronic phases (β=0.76; P=0.039). However, neither %Infarct volume nor %Hemo volume measured at
both 3 days and 56 days postreperfusion could significantly predict changes in other LV remodeling parameters (EDV, ESV, and EF).

**Discussion**

To date, the specific long-term consequence of hemorrhagic transformation of myocardial infarction in humans is unknown. Using clinical T2*-CMR, we investigated whether patients suspected of having hemorrhagic myocardial infarctions were disposed to regional iron depositions within the myocardial scar months after the infarction. Supported by the validation studies in canines, in a pilot investigation, we demonstrated that chronic iron deposition could take place in humans after acute hemorrhagic myocardial infarctions. Mass spectrometric analysis of canine myocardial tissue showed the mean iron content within the hemorrhagic infarctions to be nearly an order of magnitude greater in a chronic phase (8 weeks) after infarction compared with remote/healthy and nonhemorrhagic myocardium. In relating the mass spectrometric findings to ex vivo CMR, our noninvasive imaging studies showed that T2*-CMR can detect and quantify the extent of iron deposition within chronic hemorrhagic myocardial infarctions. These observations also provided a firm basis for noninvasively characterizing chronic iron depositions after acute hemorrhagic infarctions in canines in vivo. Volumetric CMR data from both patients and canines showed that both relative infarct and hemorrhage volume in the acute phase were significant predictors of LV remodeling on the basis of sphericity index in the chronic period after infarction. In addition, immunohistological staining for newly recruited macrophages showed an ongoing inflammatory response in the infarct tissue with iron deposits in the chronic phase of hemorrhagic infarctions.

**Detection of Regional Iron Deposition After Hemorrhagic Infarctions**

Current knowledge of pathological iron deposition in the heart has been limited to global iron overloading in the heart attributable to hematologic disorders, such as thalassemia, hemochromatosis, and sickle-cell anemia. In a number of these settings, T2* CMR has been shown to be invaluable for quantifying the global extent of iron in the heart. In particular, a recent study clearly demonstrated that the magnitude of T2* is strongly correlated with the amount of iron deposition (measured on the basis of ICP-MS) in...
postmortem hearts of deceased thalassemia patients. To date, however, there has been no direct evidence of significant localized deposition of iron in the heart. In this study, we identified reperfused hemorrhagic myocardial infarctions as the basis for a new pathogenesis associated with highly localized iron deposition in the heart that can be characterized on the basis of T2* CMR. Our findings are consistent with previous observations of the deposition of iron in other organs in the form of hemosiderin (magnetite, crystalline Fe₃O₄ particles) after the biodegradation of RBCs that are also detected on the basis of T2* MRI.

Both T2*-weighted and T2-weighted CMR techniques have been used to detect acute reperfusion intramyocardial hemorrhage. After intramyocardial hemorrhage, oxyhemoglobin in the RBCs extravasated into the myocardial interstitium is slowly converted into paramagnetic forms, such as deoxyhemoglobin and methemoglobin, in the acute phase of myocardial infarction. Our current study has shown that, in the chronic phase of infarction (ie, months after infarction), iron from the extravasated RBCs is externalized and crystallized to form the highly paramagnetic hemosiderin deposits. These paramagnetic agents induce local magnetic field inhomogeneities that appear as hypointense territories on both T2*-weighted and T2-weighted images. In the acute phase of infarction, T2-weighted techniques are also highly sensitive to elevated free water content arising from myocardial edema. Myocardial edema is a standard pathological consequence of acute I/R injury that seems hypointense on T2-weighted images. Previous studies have shown that the counteracting effects of the hypointense myocardial edema and hypointense myocardial hemorrhage in T2-weighted images can indeed obscure the detection of hemorrhage. Therefore, absolute characterization of intramyocardial hemorrhage using T2-weighted techniques may be complicated by the confounding effects of edema. In contrast, T2*-weighted techniques are relatively less sensitive to edema, making them highly sensitive to the paramagnetic effects of hemorrhage. For this reason, we chose to use a T2* mapping approach in our study to accurately characterize both acute reperfusion hemorrhage and the ensuing chronic iron depositions.

Although T2*-CMR is highly sensitive to chronic myocardial iron depositions within the infarcted territories, it is also sensitive to the magnetic susceptibility differences at the heart–lung interface (off-resonance artifacts) that manifest as hypointense regions in the inferolateral walls. Therefore, off-resonance artifacts occurring in the inferolateral walls may be misinterpreted as hemorrhagic infarctions. However, it is possible to distinguish hemorrhage from the off-resonance artifacts because hemorrhage originates in the subendocardium and is limited to the infarcted myocardium, whereas off-resonance artifacts originate at the epicardium. In this study, off-resonance artifacts were easily identified in both patients and canines, and did not limit our ability to identify hemorrhage.

Long-term Consequences of Regional Iron Deposition Within Infarcted Myocardium

The pathological consequence of regional iron deposition in the chronic phase of the hemorrhagic infarctions was also unknown before this study. In our study, immunohistochemical staining with Mac387 showed that infiltration of young macrophages is preferentially enhanced at the site of iron deposition within the infarcted myocardium. The pathological consequence of regional iron deposition in the chronic phase of the hemorrhagic infarctions was also unknown before this study. In our study, immunohistochemical staining with Mac387 showed that infiltration of young macrophages is preferentially enhanced at the site of iron deposition within the infarcted myocardium.

![Figure 5. Colocalization of newly recruited young macrophages with chronic iron deposits. Contiguous histological sections of a chronic hemorrhagic infarction stained with Perls stain and the monoclonal antibody Mac387 are shown. Newly recruited young macrophages are highly colocalized with the iron deposits throughout the infarct (GROSS). Magnified regions with (IRON+; red box) and without (IRON−; blue box) iron depositions show that young macrophages preferentially colocalize at the site iron depositions.](image1)

![Figure 6. Mass spectrometric validation of T2* cardiovascular magnetic resonance (CMR) for evaluating changes in regional myocardial iron content postreperfused infarction. A, Myocardial tissue obtained from shams, remote, Hemo−, and Hemo+ sections on day 56 postreperfusion (chronic) showed significantly higher amount of iron in Hemo+ compared with all other sections (*P<0.001). B, Linear regression analysis between ex vivo log(T2* heart) and −log([Fe] heart) showed a strong correlation (P<0.001).](image2)
chronic infarcts, with minimal or no macrophages within the infarcted territories without iron. The monoclonal antibody Mac387 detects the calcium-binding protein MRP14 that is selectively expressed by young macrophages (<7 days old) newly recruited from peripheral blood monocytes, but not mature tissue macrophages. Frangogiannis et al have used Mac387 to index for active inflammatory process in the heart. They showed that Mac387-positive cells extensively infiltrate the infarcted territories within 1 hour of I/R injury, but very few Mac387-positive cells are found after 7 days of I/R injury. Hence, our data findings here suggest that chronic iron deposition after acute reperfusion intramyocardial hemorrhage leads to an active and prolonged inflammatory process. The selective recruitment of Mac387-positive cells to the zones of chronic iron deposition within infarcted territories may be one of the potential mechanisms through which hemorrhagic infarctions lead to adverse LV remodeling. Nevertheless, additional studies are necessary to understand the relation between long-term inflammation in the presence of iron deposition and cardiac remodeling.

Iron deposition within infarcted myocardium has also been reported in the setting of iron-labeled regenerative cell therapies. It was shown that when iron-labeled stem cells are delivered to the site of infarction, even after a few weeks of delivery, the iron particulates (label) continue to persist at the site of infarction. However, the iron labels were colocalized within macrophages instead of the stem cells, suggesting that once the transplanted cells perished, the macrophages are drawn to the site of remnant iron particulates. Our data, along with the previous reports, suggest that macrophages are drawn to the site of iron regardless of its origin (endogenous or exogenous). Previous studies have also suggested that iron particulates mediate activation of microglia in the brain and differentiation of blood mononuclear cells into proinflammatory macrophages. Moreover, hemoglobin-derived iron in combination with activated macrophages has been shown to generate reactive oxygen species in chondrocytes. Although these observations have not been extended into myocardial tissue, the highly selective recruitment of young macrophages at the site of iron deposition suggests that the mechanism of macrophages recruitment to the site of chronic infarction may be similar. Because T2* of hemorrhagic infarcts seems to be stable between acute and chronic phases in both animals and patients, it seems that iron depositions from hemorrhagic infarctions are at best only slowly cleared by the macrophages. Additional studies are necessary to investigate the temporal dynamics of macrophage-based clearance of iron within infarcts. From our imaging studies in humans, it seems that the clearance takes >6 months, given that at the 6-month follow-up, significant T2* shortening was evident at the site of hemorrhagic infarction compared with nonhemorrhagic infarct territories or remote/healthy myocardium. Because T2* data were significantly correlated with direct measurements of iron in tissue (mass spectrometry analysis), clinical T2* CMR is well positioned for the assessment of changes in iron deposition in the postinfarction period.

Although our study was not performed with the goal of determining the effect of hemorrhagic infarcts on morphological or functional changes of LV, an important detail on LV remodeling emerged from this study. Our data showed that in both humans and animals with hemorrhagic infarction, LV remodeling (indexed on the basis of EDSI) between the acute and chronic phases of the infarction was significantly impacted by %Infarct and hemorrhage volume. Although the effect of infarct size on LV remodeling has been well known, our study showed that hemorrhage volume is a strong predictor of EDSI as well. In fact, the respective β coefficients from multivariable regression analyses further suggest that the percent hemorrhage (%Hemo) volume in the acute phase, in both humans and canines, has a greater influence on EDSI (β=2.02 [humans] and β=1.82 [canines]) than infarct volume in the acute phase (β=1.59 [humans] and β= 0.8 [canines]). Nevertheless, other LV remodeling parameters, such as EF, EDV, and ESV, did not reach statistical significance to show dependence on %Infarct or %Hemo volume. Further studies supported by larger sample sizes are expected to be necessary to resolve these differences.

**Limitations**

This study has certain limitations. First, our definition of hemorrhagic myocardium as the infarcted regions with low signal intensity on T2*-weighted images itself implies that the T2* values of hemorrhagic myocardium are significantly lower than those of other tissues. Nevertheless, Perls staining and mass spectrometry clearly validated that hemorrhagic myocardium, defined on the basis of low T2* values, had significantly higher iron depositions. We also showed that infarctions with low T2* values in the acute phase had concomitantly low T2* values in the chronic phase, as well.

Second, the sample size in this study was small, which may have limited the modest differences from being detected because of insufficient power. Although this could partly
explain why the impact of %Infarct or %Hemo volumes on commonly reported LV remodeling parameters, such as EDV, ESV, and EF, could not be detected, we were still able to discern a relationship between %infarct and %Hemo volume on EDSI. Moreover, further studies are necessary to evaluate the relative effects of MVO and hemorrhage on postinfarction LV remodeling.

Third, the infarction in animals was only created in the left anterior descending artery territory. Although we expect our results to hold independently of the territory of hemorrhagic infarction, additional studies are necessary to confirm whether our findings can be extended to all territories of the myocardium.

Finally, although we were able to validate our findings of iron deposition within hemorrhagic infarcts in animals on the basis of histological and mass spectrometry analysis, the evidence for iron deposition in patients with hemorrhagic infarctions is only based on T2* CMR. Nevertheless, because the CMR results between animals and patients were consistent, and because chronic iron deposition after hemorrhagic infarctions showed significant correlation against T2* CMR in animals, we anticipate that T2* CMR is a reliable alternative for the assessment of iron deposition in patients. Similarly, histopathologic findings of prolonged inflammation are limited only to hemorrhagic infarctions in canines. Additional histopathologic and mass spectrometric analyses of human hearts with chronic hemorrhagic infarctions may be necessary to rigorously extend these findings to humans.

Conclusion

Hemorrhagic myocardial infarction can lead to iron depositions within the infarct zones, which can be a source of prolonged inflammatory burden in chronic phase of myocardial infarction.

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References


CLINICAL PERSPECTIVE

Adverse left ventricular remodeling after myocardial infarction infarction culminating in heart failure is one of the growing causes of morbidity and mortality in our society. This work provides a new pathophysiological phenomenon linking acute myocardial injury and chronic left ventricular remodeling. For the first time, we demonstrate that acute hemorrhage leads to localized iron deposition and that such remnants prolong inflammation into the chronic phase of the infarction. We also show that T2* cardiovascular magnetic resonance, which is already used in the clinical setting, can be used to noninvasively characterize the chronic iron burden on myocardial tissue. We envision that reperfusion hemorrhage, iron deposition, and the inflammatory-burden axis may evolve as an important mechanism of postinfarction remodeling. The present work lays the ground for future research in basic, translational, and clinical science, where the pathological iron deposition may represent a new therapeutic target in acute myocardial infarction.
Chronic Manifestation of Postreperfusion Intramyocardial Hemorrhage as Regional Iron Deposition: A Cardiovascular Magnetic Resonance Study With Ex Vivo Validation
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SUPPLEMENTARY MATERIALS AND METHODS

CMR STUDIES IN PATIENTS

Patients were enrolled in the study following informed consent and studied according to the protocols approved by the Institutional Review Board of Foothills Medical Center (Calgary, AB, Canada). Patients with acute ST-elevation myocardial infarction (n = 15; 3 women; mean age = 58±8 years) meeting American Heart Association diagnostic criteria were enrolled in the study only if successful percutaneous coronary intervention (PCI), defined as a TIMI flow grade of 3 post-PCI, was performed within 12 hours of the onset of symptoms. Patients were excluded from the study if they had previous myocardial infarctions or had contraindications for a Cardiovascular Magnetic Resonance (CMR) study. All enrolled patients underwent CMR 3 days (acute) after successful PCI and again 6 months (chronic) after initial enrollment.

All patient imaging studies were performed on a clinical 1.5T MRI system (MAGNETOM Avanto, Siemens Medical Solutions, Erlangen, Germany) equipped with high-performance gradient system (maximum gradient amplitude of 45 mT/m and maximum slew rate of 200 T/m/s). B_1 field was transmitted using the scanner’s integrated body coil and an eight-channel flexible phased-array coil was placed on the chest for signal reception. All anatomical axes were localized and a volume-selective shim covering the whole heart was performed. Contiguous short-axis sections covering the entire LV along with 2, 3 and 4 chamber long-axis views of the heart were acquired at mid-diastole using cine-SSFP¹, Multi-gradient echo²³ (T2*-weighted) and LGE imaging⁴. Typical imaging parameters used for cine-SSFP images were TR / TE = 3.32 / 1.16ms, flip angle = 65°, BW = 930 Hz/pixel, 25 cardiac phases, FOV = 340 mm x 276 mm, imaging matrix = 192 x 156, imaging section thickness = 10 mm and number of averages = 1. Typical imaging parameters used for T2*-weighted images were TR = 240 ms, 6 echoes with TEs = 2.6, 4.8, 7.0, 9.3, 11.5 and 13.7ms, flip angle = 10°, BW = 355 Hz/pixel, FOV = 420 mm x 328 mm, imaging matrix = 256 x 200, slice thickness = 10 mm and number of averages = 1. LGE images
were acquired 10-15 minutes after an intravenous Gadolinium-DTPA administration (0.2 mmol/kg of body weight) using an optimal TI to suppress signal from remote myocardium. An IR-prepared fast low angle shot (FLASH) sequence was employed with the typical imaging parameters being TR = 1 R-R interval, TE = 3.32 ms, flip angle = 20°, BW = 235 Hz/pixel, FOV = 400 mm x 300 mm, imaging matrix = 256 x 192, slice thickness = 10 mm and number of averages = 1. FOVs were rectangular and no image acceleration method was used.

**PROOF-OF-CONCEPT STUDIES IN CANINES**

**Animal Preparation**

Canines (n = 23, 20-25 kg) were enrolled and studied according to the protocols approved by the Institutional Animal Care and Use Committee. Each dog was given an intramuscular injection of the preanesthetic tranquilizer Innovar (0.4 mg/ml of fentanyl and 20 mg/ml of droperidol) at a dose of 1 ml / 25-50 kg of body weight. Subsequently, the dog was anesthetized with an intravenous injection of Propofol (5.0-7.5 mg/kg), endotracheally intubated and maintained on gas anesthesia (2.0-2.5% isoflurane with 100% oxygen). Animals were artificially ventilated at 1-2 L/min with the respiration rate being continuously adjusted to maintain partial pressure of CO₂ in blood (PaCO₂) between 30 and 35 mmHg. Left lateral thoracotomy was performed at the fourth intercostal space, and the exposed heart was suspended in a pericardial cradle. Aortic and left atrial catheters were inserted and secured for invasive blood pressure monitoring and drug delivery. A portion of the proximal left anterior descending artery (LAD) was isolated and a hydraulic occluder was looped around the vessel 1.0-1.5 cm distal to the bifurcation of left main coronary artery⁵ (Fig.S1). A Doppler ultrasound flow probe (Crystal Biotech, Northborough, MA, USA) was circumferentially secured 2.0-2.5 cm downstream from the occluder to verify the fidelity of occlusion. Systemic O₂ saturation, PaCO₂, body temperature, blood pressure,
respiration rate, heart rhythm and rate were continuously monitored throughout the surgery. The chest was closed and the dog was allowed to recover for 7 days prior to ischemia-reperfusion (I/R) injury.

**Inducing Ischemia-Reperfusion Injury**

On day 7 post-surgery, each dog was anesthetized and reversible LAD stenosis was induced by gently forcing saline through the open end of the occluder tubing using a micro-push syringe (250μl GASTIGHT syringe, Hamilton Company, Reno, NV, USA) resulting in the constriction of LAD. The extent of LAD occlusion was continuously monitored by Doppler flow velocities. After achieving complete LAD occlusion, the ballooned tubing was clamped and held for 3 hours. At the end of 3 hours of ischemia, reperfusion was established by releasing the clamp and completely drawing out the saline from the tubing. Vital parameters, similar to those described earlier for the surgical procedure, were continuously monitored. To minimize fatal ventricular arrhythmias from I/R injury, all dogs were pre-treated with Amiodarone (200 mg/day, TEVA Pharmaceuticals USA, Sellersville, PA, USA) for 2 weeks prior to I/R injury. Arrhythmias occurring during the ischemia and reperfusion were controlled by intravenous injection of 1-2 ml lidocaine (20 mg/ml) as needed.

**EXPERIMENTAL GROUPS**

All animals were broadly divided into two groups – Shams (n = 3) and Infarct (n = 20). The Sham group underwent the surgical procedure, but was not subjected to I/R injury. The Infarct group underwent 3 hours of no-flow ischemia followed by reperfusion. All the three Sham animals survived the entire studies. From the Infarct group, four dogs died within the first 2 hours of establishing reperfusion (despite resuscitation efforts) and in two dogs, reperfusion was not established due to the failure of occluder implementation. These dogs were completely excluded from the study. From the remaining fourteen dogs that were successfully subjected to I/R injury, three dogs were sacrificed on day 3 post I/R injury for histological validation of acute hemorrhagic infarctions. The remaining eleven dogs from the Infarct
group were studied up to day 56 post I/R injury. The results presented in this paper (except histological
evidence for acute hemorrhagic infarctions) were obtained from these remaining 11 dogs of the Infarct
group and 3 dogs of the Sham group that were sacrificed immediately following the chronic phase CMR
study.

*In-vivo CMR studies*

Animals from the Infarct group underwent CMR on days 3 (acute) and 56 (chronic) post-I/R injury, while
the Shams underwent CMR at similar time points. All CMR studies were performed on a clinical 1.5T
magnetic resonance imaging (MRI) system (MAGNETOM Espree, Siemens Medical Solutions, Erlangen,
Germany) equipped with a high-performance gradient system (maximum gradient amplitude of 40 mT/m
and maximum slew rate of 200 T/m/s). The animals were anesthetized, intubated and ventilated as
described before for the surgical procedure. They were placed on the scanner table in feet-first, right
anterior position. A flexible eight-channel phased-array surface coil was placed on their chests for signal
reception. B1 field changes were transmitted using the scanner’s integrated body coil. Scout images were
acquired to localize the heart and a volume-selective shim covering the whole heart was performed to
minimize off-resonance artifacts. Multiple cardiac-gated breath-held 2D images of contiguous short-axis
sections covering the entire left-ventricle (LV) and the three long-axis views (2 chamber, 3 chamber and 4
chamber) were acquired using cardiac phase resolved SSFP (cine-SSFP)1, T2*-weighted imaging2-3 and
Late Enhancement (LGE) imaging4. Multi-gradient echo (T2*-weighted) and LGE images were acquired
at mid-diastole when the cardiac motion is minimal. Cardiac gating was achieved by using prospective
ECG triggering and breath-holding was achieved by suspending ventilation at end-expiration. Anesthesia
was carefully controlled during breath-holding to avoid any spontaneous breathing. A 2-3 minute rest
period between successive breath-holds was given to maintain the heart rate at a constant level throughout
all acquisitions.
Typically used cine imaging parameters were TR / TE = 3.5 / 1.75 ms, flip angle = 70°, 25-30 cardiac phases per imaging section and readout bandwidth (BW) = 930 Hz/pixel. Significant wall motion abnormalities were observed in the LAD territories of infarcted dogs both during acute and chronic phase CMR studies. Acute hemorrhage and chronic iron loading were evaluated using T2*-weighted images acquired by a multiple gradient-echo sequence. Typically used imaging parameters were TR = 220 ms, 12 echoes with TEs = 3.4, 6.4, 9.4, 12.4, 15.4 and 18.4 ms, flip angle = 12° and BW = 566 Hz/pixel. All in-vivo imaging studies were terminated with the acquisition of Phase-Sensitive Inversion Recovery LGE images using a non-selective inversion recovery (IR) prepared SSFP sequence. Initially, 0.2 mmol/kg of Gadolinium-DTPA contrast agent (Magnevist, Bayer Healthcare Pharmaceuticals Inc., Wayne, NJ, USA) was administered intravenously using a power injector followed by a 10 ml saline flush. An optimal inversion time (TI) to null the apparent normal myocardium was then determined from TI scout images. LGE images were acquired 10-15 minutes after contrast administration using the following imaging parameters: TR / TE = 3.5 / 1.75 ms, flip angle = 40° and BW = 1002 Hz/pixel. Other commonly used imaging parameters for all the scans were Field-of-view (FOV) = 166 mm x 280 mm, imaging matrix = 116 x 192, slice thickness = 8 mm and number of averages = 1. FOV was rectangular for all the scans. No image acceleration methods were used.

All animals in the Infarct group sustained acute hemorrhagic infarctions as indicated by the acute phase T2*-weighted and LGE images. All animals in the Sham group did not sustain any myocardial infarction throughout the study as verified by both acute and chronic phase LGE images.

**Ex-vivo CMR studies**

Animals were euthanized immediately after the chronic phase CMR study by intravenously administering 0.2 ml/kg body weight of Euthasol (390 mg/ml sodium pentobarbital and 50 mg/ml phenytoin sodium) and their hearts were excised. Each heart was manually sliced into 1cm thick slices along the LV short-axis. Each slice was immersed in 0.05M Phosphate-buffered saline (PBS; pH = 7.4) and ex-vivo 2D T2*-
weighted and Phase-Sensitive Inversion Recovery LGE images were acquired using a multiple gradient-echo sequence and IR-prepared SSFP sequence respectively. A head coil was used for signal reception for ex-vivo imaging. Typical imaging parameters used for ex-vivo T2*-weighted and LGE images were the same as those used for the corresponding in-vivo T2*-weighted and LGE images. The imaging slice was carefully selected to avoid any partial-voluming between the myocardial tissue and the PBS bath. FOV was rectangular and no image acceleration method was used.

GROSS HISTOLOGICAL IDENTIFICATION OF MYOCARDIAL INFARCTS

Ex-vivo myocardial slices from every animal were stained with triphenyl tetrazolium chloride (TTC)\textsuperscript{7} to histochemically validate irreversible myocardial damage and delineate the infarcted territories from the viable myocardium. TTC stained viable myocardium brick-red as membrane-bound dehydrogenases and other cofactors reduce the tetrazolium salts to a brick-red formazan pigment, while infarcted myocardium remains unstained. Briefly, the slices were incubated in 1\% (w/v) TTC in PBS at 37°C for 15-20 minutes and photographed under room light. Chronic iron overloading appeared yellowish-brown within the pale infarcted territories. All dogs from the Infarct group contained a number of slices with TTC-unstained infarcted regions within the LAD territory of LV. Few slices were negative for infarction and were discarded. All slices from the Sham dogs were negative for infarction.

IMAGE ANALYSIS

\textit{Semi-automatic in-vivo image analysis}

All in-vivo image analyses (both acute and chronic from animals and patients) were performed off-line using a validated and certified cardiac MR image processing software (cmr\textsuperscript{42}, Circle Cardiovascular Imaging Inc., Calgary, AB, Canada; Fig. S2). To minimize unwanted off-resonance\textsuperscript{8} and flow artifacts,
in-vivo T2* maps were constructed by fitting the multi-echo data to a mono-exponential decay (Fig.S3). Endocardial and epicardial contours were drawn for each imaging slice on the cine-SSFP image corresponding to the appropriate mid-diastolic phase. The contours were then copied on to both T2*-weighted and LGE images and adjusted when necessary. Remote myocardium was identified as the region showing no hyperintensity on LGE images. A reference region-of-interest (ROI) was drawn in the remote myocardium and a threshold based semi-automatic method was used to detect infarcted myocardium on LGE images. Infarcted myocardium was defined as the hyperintense region on LGE images with ≥10 adjacent pixels having mean signal intensity (SI) 5 standard deviations (SD) greater than the mean SI of reference ROI. In the final analysis of infarcted myocardium on LGE images, regions of hypointense territories (microvascular obstruction) within the hyperintense territories were manually included.

The reference ROI from the LGE image was copied on to the T2*-weighted image acquired at the longest TE among all the echoes used to construct the T2* map, i.e. TE = 18.4 ms for animals and TE = 13.7 ms for patients. Hemorrhagic myocardium was identified on this image as the hypointense region with ≥10 adjacent pixels having mean SI at least 2 SDs below the mean SI of the reference ROI. While drawing the reference ROI and in the final analysis of the hemorrhagic myocardium, care was taken not to include regions affected by off-resonance artifacts arising from susceptibility shifts at the heart-lung interface. Also, any hypointense region lying outside the infarcted territory was excluded from the analysis.

**Classification of in-vivo imaging sections**

For animals from the Infarct group, all in-vivo imaging sections were divided into three different groups based on in-vivo LGE and T2*-weighted images. Myocardial imaging slices that contained hyperintense LAD infarct regions on LGE images with a hemorrhagic core on the corresponding T2*-weighted images were classified as hemorrhagic infarct sections. Similarly, imaging slices that contained LAD infarct regions on LGE images but no hemorrhagic core on the corresponding T2*-weighted images were
classified as non-hemorrhagic infarct sections. Imaging slices that did not contain any infarcted regions were not used for further analysis.

For animals from the Sham group, T2* values were measured from the entire myocardium. Mean per-slice in-vivo T2* values were measured for hemorrhagic infarct (Hemo+), non-hemorrhagic infarct (Hemo-), remote myocardium (Remote) and sham myocardium (Sham) from the corresponding T2* maps (in-vivo T2*_slice). Also, mean whole-heart in-vivo T2* values were measured for each heart from Hemo+, Hemo-, Remote and Sham groups by averaging across the corresponding imaging slices (in-vivo T2*_heart).

For patients, a similar classification of in-vivo imaging slices was used and in-vivo T2* values on a per-section and whole-heart basis were measured for Hemo+, Hemo- and remote groups (no shams) from the corresponding T2* maps.

**Semi-automatic ex-vivo image analysis and classification**

Ex-vivo image analysis was also performed offline using cmr42. T2* maps for each ex-vivo myocardial slice from the canines were constructed by fitting multi-echo data from only the first 6 echoes (TEs 3.4-18.4ms) to a mono-exponential decay. Remote myocardium was defined as the region stained brick-red by TTC along with the absence of hyperintensity on ex-vivo LGE image. A reference ROI was drawn within the remote myocardium on LGE image and infarcted myocardium was defined as the region with ≥10 adjacent pixels having mean SI at least 5 SDs above the mean SI of the reference ROI. Subsequently, the reference ROI was copied on to the T2*-weighted image acquired at TE=18.4ms. Hemorrhagic myocardium was defined as the region with ≥10 adjacent pixels having a mean SI at least 2 SDs below the mean SI of the reference ROI.

On the basis of ex-vivo LGE and T2*-weighted images, as well as corresponding TTC staining, all ex-vivo myocardial slices were also classified as sham, hemorrhagic infarct or non-hemorrhagic infarct slices as earlier (refer to in-vivo image analysis). Excellent correlation between TTC-unstained infarcted region and hyperintense infarcted region on LGE images was observed. Slices from the infarcted dogs that did
not contain TTC-unstained infarcted regions or hyperintense regions on corresponding ex-vivo LGE images were discarded. Mean per-slice ex-vivo T2* values for hemorrhagic infarcts (Hemo+), non-hemorrhagic infarcts (Hemo-), remote myocardium (Remote) and sham slices (Sham) were measured from T2* maps (ex-vivo T2*_slice). Also, mean whole-heart ex-vivo T2* values for each dog were measured for Hemo+, Hemo-, Remote and Sham groups by averaging across all the corresponding slices (ex-vivo T2*_heart).

**Left-ventricular morphological and functional analyses**

Infarct volume and hemorrhage volume were calculated with respect to the total LV volume (%Infarct and %Hemo respectively). LV mass, end-diastolic volume (EDV), end-systolic volume (ESV), ejection fraction (EF) and end-diastolic sphericity index (EDSI) were calculated from Cine-SSFP images. All measurements were normalized to the body surface area calculated using the below formulae.

\[
\text{For humans, Body Surface Area (m}^2) = \frac{\sqrt{\text{Height (cm) \times Weight (kg)}}}{3600}
\]

\[
\text{For canines, Body Surface Area (m}^2) = 10.1 \times (\text{Weight (g)})^{0.67} \times 10^{-4}
\]

**ISOLATION OF TISSUE SAMPLES**

From both ex-vivo hemorrhagic and non-hemorrhagic infarct slices, unstained TTC sections (only from the densely infarcted areas) were carefully cut out. Care was taken not to cut into the infarct border zone or any surrounding TTC-stained viable myocardium. To facilitate further tissue analysis, all blocks of hemorrhagic and non-hemorrhagic infarcts were further cut into their constituent smaller hemorrhagic (Hemo+) and non-hemorrhagic (Hemo-) infarct samples (0.5-0.8cm³). Similarly, from each hemorrhagic and non-hemorrhagic infarct slice, at least 2 samples of TTC-stained viable myocardium were cut out (Remote). From each sham slice, at least 2 samples of TTC-stained viable myocardium were cut out.
(Sham). In total, we have analyzed 55 samples of remote, 20 samples of Sham, 31 samples of Hemo- and 183 samples of Hemo+ of myocardial tissue.

HISTOLOPATHOLOGIC STUDIES

A representative myocardial sample from each of the Hemo+, Hemo-, Remote and Sham groups was obtained from every animal. The sample was dehydrated, embedded in a paraffin block and four contiguous 5µm sections were obtained using a microtome. The four sections were stained with regressive Hematoxylin and Eosin (H & E), Masson’s Trichrome, Perl’s and monoclonal antibody Mac387 (for newly recruited macrophages; Dako Carpinteria, CA) stains respectively using standard techniques. The sections were mounted on glass slides and scanned at 100X magnification using an ACIS II technology based ChromaVision digital slide scanner (Clarient Inc., Aliso Viejo, CA, USA). The slides were also imaged at 400X magnification using an Olympus BX41 stereo compound microscope with dual view side (Olympus America Inc., Center Valley, PA, USA).

H & E staining was used to distinguish between necrotic and viable myocardium. Hematoxylin stained nuclei of viable cells blue, while eosin stained the cytoplasmic structures pinkish red. Acute infarcted myocardium showed massive infiltration of inflammatory cells. Extravasated red blood cells (eosinophilic structures) in hemorrhagic infarctions were stained intensely red by eosin. Chronic infarcted myocardium was stained faint pink with no distinctly visible individual cells.

Masson’s trichrome staining was used to identify collagen deposition within the infarcted myocardium. Viable myocardium was stained dark red, while collagenous scar was stained intensely blue. Perl’s staining was used to visually identify iron deposition within the infarcted myocardium. Iron deposits externalized from the extravasated red blood cells were stained blue, while cells and cytoplasmic structures were stained pink. The monoclonal antibody Mac387 was used to identify newly recruited macrophages (dark brown) within the infarct.
LOCAL IRON DEPOSITION MEASUREMENTS

The extent of iron deposition ([Fe]) within each myocardial sample from the Hemo+, Hemo-, Remote and Sham groups was analyzed using Inductively Coupled Plasma – Mass Spectrometry (ICP-MS). The samples were briefly rinsed with ultrapure double-distilled deionized (Milli-Q) water (resistivity of 18MΩ-cm at 25°C), blotted, weighed and placed in individual autoclavable Teflon centrifuge tubes (Thermo-Fisher Scientific, Waltham, MA, USA). The Teflon tubes were soaked in 3% nitric acid overnight and rinsed with Milli-Q water before use. 2ml of 69% (w/v) trace-metal grade nitric acid (GFS Chemicals Inc., Columbus, OH) was added to the samples and vented. The samples were then microwave digested using a Milestone EthosEZ closed microwave digestion system (Milestone S.r.l., Bergamo, Italy) equipped with temperature and pressure sensors (maximum temperature of 260°C and maximum pressure of 10 MPa). The digestion temperature was ramped up at 12°C/min and maintained at 120°C for 10 minutes before allowing to cool down to room temperature. The digested samples were then filtered through 0.45µm Teflon syringe-filters (Thermo-Fisher Scientific, Waltham, MA, USA) and the filtrates were collected in individual 15ml metal-free polypropylene tubes (VWR International Inc., Bridgeport, NJ). The filtrates were diluted to 1:40 of original concentration with Milli-Q water and an internal standard mixture (CPI International, Santa Rosa, CA, USA) containing Sc, Tb, Y, In and Bi was added. A set of standards with concentrations ranging from 0ppb to 100ppb was prepared using a mixed element solution (CPI International, Santa Rosa, CA, USA). All samples and standards were prepared in duplicates in a 2% nitric acid matrix.

All samples were analyzed on a quadrupole based X Series 2 ICP-MS (Thermo-Fisher Scientific, Waltham, MA, USA) equipped with Collision Cell Technology to reduce interference from doublets. Samples were introduced into the ICP-MS at a rate of 0.5ml/min using an automated SC-FAST system (Elemental Scientific Inc, Omaha, NE, USA) comprising of an autosampler, diaphragm vacuum pump, PFA-ST nebulizer and a Peltier-cooled cyclonic spray chamber. Data was acquired using the dedicated PlasmaLab software. Fe content measured within each sample was averaged between the two duplicates.
and expressed as μg of Fe per g of sample ([Fe]_{sample}). Also, whole-heart Fe content ([Fe]_{heart}) was measured by weight-averaging [Fe]_{sample} of their constituent samples.
SUPPLEMENTARY FIGURES

Figure S1. Isolation of left anterior descending (LAD) artery for the placement of hydraulic occluder.
Figure S2. Semi-automatic threshold-based analysis of images acquired from an infarcted dog during acute phase (day 3) CMR studies. (A) Representative Late enhancement (LGE) image showing gadolinium hyperenhanced infarcted region (red arrows) with microvascular obstruction (MVO; dark region) enclosed within. (B) Infarcted myocardium (highlighted yellow pixels on the LGE image from (A)) identified as the hyperintense region with mean signal intensity (SI) 5 standard deviations (SDs) greater than that of reference ROI (blue ROI). MVO (orange region) was included in the final analysis of infarcted myocardium. (C) Representative T2*-weighted image acquired at TE=18.38ms showing hypointense hemorrhagic myocardium (red arrows). (D) Hemorrhagic myocardium (highlighted blue pixels on the T2*-weighted image from (C)) identified as the region with mean SI 2 SDs lesser than that of reference ROI (blue ROI). Region affected by off-resonance artifacts (white arrow) was not included in the analysis. (E) Color-coded T2* map showing the hemorrhagic myocardium (bright red region within a red ROI). Region affected by off-resonance artifact (white arrow) was excluded.
Figure S3. Monoexponential fits of multi-echo data from T2*-weighted images. Representative monoexponential fits for Sham (T2*=42.1ms), Remote (T2*=40.7ms), Hemo+ (T2*=19.1ms) and Hemo- (T2*=39.6ms) myocardium are shown.
SUPPLEMENTARY REFERENCES


9. Bondarenko O, Beek AM, Hofman MB, Kuhl HP, Twisk JW, van Dockum WG, Visser CA, van Rossum AC. Standardizing the definition of hyperenhancement in the quantitative assessment of
