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Manganese-enhanced T_1 mapping in the myocardium of normal and infarcted hearts

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Running Title: MEMRI of Normal and Infarcted Myocardium

Abstract

Background

Manganese-enhanced MRI (MEMRI) has the potential to identify viable myocardium and quantify calcium influx and handling. Two distinct manganese contrast media have been developed for clinical application, mangafodipir and EVP1001-1, employing different strategies to mitigate against adverse effects resulting from calcium-channel agonism. Mangafodipir delivers manganese ions as a chelate and EVP1001-1 co-administers calcium gluconate. Using myocardial T_1 mapping, we aimed to explore chelated and non-chelated manganese contrast agents, their mechanism of myocardial uptake, and their application to infarcted hearts.

Methods

T_1 mapping was performed in healthy adult male Sprague-Dawley rats using a 7T MRI scanner before and after non-chelated (EVP1001-1 or $MnCl_2$ [22 µmol/kg]) or chelated (mangafodipir, [22-44 µmol/kg]) manganese-based contrast media in the presence of calcium channel blockade (diltiazem, 100-200 µmol/kg/min) or sodium chloride (0.9%). A second cohort of rats underwent surgery to induce anterior myocardial infarction by permanent coronary artery ligation, or sham surgery. Infarcted rats were imaged with standard gadolinium delayed-enhancement MRI (DEMRI) with inversion recovery techniques (DEMRI inversion recovery) as well as DEMRI T_1 mapping. A subsequent MEMRI scan was performed 48 h later using either

non-chelated or chelated manganese and T_1 mapping. Finally, animals were culled at 12 weeks and infarct size was quantified histologically with Masson's trichrome (MTC).

Results

Both manganese agents induced concentration-dependent shortening of myocardial T_1 values. This was greatest with non-chelated manganese, and could be inhibited by 30-43% with calcium-channel blockade. Manganese imaging successfully delineated the area of myocardial infarction. Indeed, irrespective of the manganese agent there was good agreement between infarct size on MEMRI T_1 mapping and histology (bias 1.4%, 95% CI -14.8 to 17.1 P>0.05). In contrast, DEMRI inversion recovery overestimated infarct size (bias 11.4%, 95% CI -9.1 to 31.8 P=0.002), as did DEMRI T_1 mapping (bias 8.2%, 95% CI -10.7 to 27.2 P=0.008). Increased manganese uptake was also observed in the remote myocardium, with remote myocardial ΔT_1 inversely correlating with left ventricular ejection fraction post myocardial infarction (r = -0.61, P = 0.022).

Conclusions

MEMRI causes concentration and calcium channel-dependent myocardial T_1 shortening. MEMRI with T_1 mapping provides an accurate assessment of infarct size and can also identify changes in calcium handling in the remote myocardium. This technique has potential applications for the assessment of myocardial viability, remodelling and regeneration.

Keywords

Manganese-enhanced MRI, delayed-enhancement MRI, DEMRI, MEMRI, T_1 mapping, myocardial viability, cardiomyopathy, myocardial calcium-handling.

Introduction

With major and sustained advances in imaging techniques over the past 3 decades, magnetic resonance imaging (MRI), along with other advanced modalities such as positron emission tomography (PET), has become an essential element to non-invasive structural and functional cardiac imaging [1,2,3]. Current standard clinical methods use inversion recovery delayed enhancement sequences after gadolinium-based contrast administration to image myocardial scar. This allows assessment of viability by assessing the transmural extent of myocardial scar and is used to predict prognosis, guiding the appropriateness of coronary revascularization [4], with excellent reproducibility [5]. Although an invaluable tool in viability assessment, the non-selective passive extracellular redistribution of gadolinium is unable to characterise and define viable myocardium directly [6]. Indeed, quantification by gadolinium delayed enhancement MRI (DEMRI) is subject to overestimation of acute infarct size due to tissue oedema [7]. It is also associated with imaging artefact and interpretation bias in challenging patient populations. Furthermore, whilst significant advances have enabled multiparametric MRI assessment of gadolinium distribution and dynamics to help determine aetiology [8], in practice the different patterns of late enhancement are neither completely specific nor sensitive for different forms

of cardiac pathology where significant overlap is seen, as with aortic stenosis and cardiac sarcoidosis [9,10,11].

There has been increasing interest in a range of alternative contrast media [12] to broaden the capabilities and functional assessments of MRI. Manganese, a paramagnetic calcium analogue, enters active cardiomyocytes via voltage-gated calcium-channels, increasing MRI-detectable T_1 relaxivity [13]. As such, manganese-enhanced MRI (MEMRI) has the potential to quantify calcium influx and handling directly, and to identify functional cardiomyocytes actively cycling calcium. Manganese-based contrast media can exist in either chelated (e.g. mangafodipir, manganese dipyridoxyl diphosphate, Mn-DPDP), or non-chelated forms (e.g. EVP1001-1, manganese gluconate with calcium gluconate [14]) and their uptake appears predominantly dependent on the activity of voltage-gated calcium channels during excitation-contraction coupling [15,16,17]. The different formulations of these manganese contrast media reflect different strategies to address adverse effects of manganese resulting from calcium-channel agonism which would otherwise be prohibitive to clinical use. Mangafodipir delivers manganese ions in the form of a chelated agent, similar to conventional MRI contrast agents, resulting in a lower effective circulating dose of manganese ions. Conversely, EVP1001-1 utilises co-administration of calcium, in the form of calcium gluconate, to negate toxicity. Both techniques have established safety and tolerability in clinical studies as well as efficacy in MRI contrast imaging.

Whilst short term cardiac safety of intravenous $MnCl_2$ has been suggested in a pilot study of 15

healthy volunteers at an equivalent molar dose [18], due to the risk of acute toxicity in cardiac patients, there is no expectation that $MnCl_2$ be developed further for clinical utility. However, given that established clinical safety has been demonstrated for both EVP1001-1 and mangafodipir at doses required for cardiac MRI, there is widespread application for both mangafodipir and EVP1001-1, notably for cardiac imaging. Preclinical studies with mangafodipir and $MnCl_2$ in healthy myocardium and EVP1001-1 in myocardial infarction models have described myocardial T_1 shortening properties [19] and demonstrated favourable agreement with histological infarct assessment [20]. Moreover, recent pre-clinical studies have suggested that MEMRI can lead to better infarct discrimination and the identification of viable myocardium [21] as well as the ability to assess engraftment of myocardial stem cells [22].

Despite longstanding knowledge of the paramagnetic properties of manganese, the development and clinical translation of manganese contrast agents has been limited by early issues with toxicity and the subsequent widespread utility of gadolinium agents which have since dominated clinical use in the field. More recently, with concerns about neurological accumulation of some gadolinium agents [23,24,25], and as problems with acute manganese toxicity have been overcome, there is scope to revisit this agent with high potential in cardiac imaging.

Given the potential benefits of MEMRI, we aimed to compare myocardial enhancement using chelated and non-chelated manganese-based contrast media, and to determine the contribution of calcium-channels to their uptake. This study represents a novel head-to-head

comparison of three manganese contrast agents, using T_1 mapping to assess and compare their respective T_1 shortening properties, utility and accuracy in quantifying myocardial infarction as compared to DEMRI (inversion recovery and T_1 mapping) and histological analysis, and explore altered calcium-handling in remodelling myocardium. This preclinical work is crucial to inform clinical translation and further development of the potential of MEMRI in myocardial viability assessment.

Methods

All studies were approved by the University of Edinburgh Animal Welfare and Ethical Review Body and were carried out in accordance with the UK Home Office Animals (Scientific Procedures) Act 1986. Male Sprague Dawley rats (250-400g, n=55) were purchased from Charles River Ltd (Haddington, UK) and housed, with free access to food and water, in the Central Bioresearch Services, University of Edinburgh for 7 days prior to use in the study.

Magnetic Resonance Imaging

All MRI experiments were performed using a 7T horizontal bore NMR spectrometer (Agilent Technologies, Yarnton, UK), equipped with a high-performance gradient insert (120 mm inner diameter), maximum gradient strength 400 mT/m. Rats were anaesthetised with 1.5-2% isoflurane (Zoetis Ltd., London UK) in oxygen/air (50/50, 1 L/min) with subsequent cannulation of the tail vein for drug/contrast agent administration. The animals were secured in a cradle

(Rapid Biomedical GmbH, Rimpar, Germany). The heart rate, respiration rate, and rectal temperature were monitored (Model 1030 monitoring and gating system, Small Animal Instruments Inc. Stony Brook, NY, USA), with body temperature maintained at 37°C by a heat fan. A 72-mm quadrature volume coil was used for transmission with signal reception by a four-channel phased array coil (Rapid Biomedical GmbH, Rimpar, Germany).

Scout images were taken to confirm correct positioning and to orientate 9x2mm axial slices from the aortic valve annulus to the apex, perpendicular to the interventricular septum (short axis slices). The slice plan was carefully replicated between scans by ensuring the same slice plan methodology, which was agreed by two operators at the time of scanning to agree adequate orientation. Selection was then made of the mid-ventricle short axis slice for further interrogation with cine and the T_1 mapping sequence. Long-axis cines and a short-axis stack were acquired (to allow left ventricular ejection fraction calculation), with a cardiac-gated gradient echo imaging (TR=1×R-R interval; TE=1.4ms; flip angle=15°; FOV=50×50mm^2; matrix=128×128; slice thickness=1.5mm).

T_1 mapping for calculation of regional left ventricular myocardial T_1 relaxation times was accomplished using a gradient-echo, cardiac-gated Modified Look-Locker Inversion recovery sequence (MoLLI) [26] whereby 14-20 images were acquired at unique inversion times (dependent on heart rate, ranging from approximately 0.20 to 3.00s) with the $TR_{inversion}$>3xT_1 of myocardium ($TR_{inversion}$>4.50s). Imaging readout was with a cardiac fast gradient echo (TR=3.50ms; TE=1.77ms; flip angle=10°, matrix 128×128; ETL=8; FOV=50×50mm^2; in-plane

resolution=0.39×0.39mm^2; trigger delay=1xR-R; slice thickness=2mm; 8 signal averages) to compensate for respiratory motion.

Manganese Enhanced MRI of Healthy Rat Myocardium

EVP1001-1 (SeeMore™, Eagle Vision Pharmaceuticals Corporation, Downingtown, PA, USA) was administered as an intravenous bolus at the manufacturer's recommended dosage of 22 µmol/kg manganese over 3-4 min. Mangafodipir (Teslascan™, IC Targets AS, Oslo, Norway) was similarly administered as a bolus of 22 or 44 µmol/kg manganese over 3-4 min. Manganese chloride solution ($MnCl_2$) was prepared using $MnCl_2 \bullet 4H_2O$ (Sigma-Aldrich Ltd, Gillingham, UK) and sterile water (Sigma-Aldrich Ltd, Gillingham, UK), 22 µmol/kg manganese administered over 3-4 min. All manganese contrast media were delivered in volumes of 2.2 mL/kg, diluted with 0.9% saline solution (Sigma-Aldrich Ltd, Gillingham, UK), to maintain the rate of manganese delivery constant between agents.

Diltiazem (Sigma-Aldrich Ltd, Gillingham, UK) diluted with 0.9% saline solution was infused at 100 µmol/kg/min intravenously, increased to approximately 120-200 µmol/kg/min until a satisfactory chronotropic response was achieved (reduction of >10% in heart rate) or the upper limit was reached. Infusion was commenced approximately 10 min prior to T_1 mapping to ensure stable and adequate heart rate response. Control administrations consisted of a similar volume (8 mL/kg) of 0.9% saline over 180 min. Administration of all agents was followed by a further saline flush of 0.4 mL to ensure complete delivery to the circulation accounting for dead space in the fine bore intravenous line. T_1 mapping was performed in all animals at baseline and

198 then at approximately 5, 20, 40 and 60 min post-manganese contrast media administration,
199 while cine imaging was performed in a cohort of animals at approximately 15, 30 and 50 min
200 post contrast. The time intervals were determined by technical considerations relating to the
201 length of time required for the T_1 mapping sequences.
202
203 **Myocardial Infarction Model**
204 Rats were anaesthetised with isoflurane (5% in 1.5 L/min oxygen for induction), followed by
205 intraperitoneal ketamine 100 mg/kg (Zoetis Ltd, London, UK) and medetomidine 1 mg/kg (Orion
206 Pharma, Espoo, Finland) for maintenance anaesthesia. Buprenorphine 0.05 mg/kg (Alstoe Ltd,
207 York, UK) was administered immediately before and 24 hours post-operatively for analgesia.
208 Tracheal intubation was achieved under direct vision and ventilation was maintained with a
209 rodent ventilator (Harvard Apparatus Model 683, MA, USA, tidal volume 2.5 cm^3, respiratory
210 rate 60/min).
211
212 Myocardial infarction was induced as we have previously described [27]. Briefly, the skin was
213 incised at the level of the left third and fourth ribs where the pectoral muscles were divided and
214 retracted. Left lateral thoracotomy was then performed. With minimal handling, the
215 pericardium was ruptured and the heart gently exteriorised from the thorax and a non-
216 absorbable 5-0 ligature was placed around the left anterior descending coronary artery just
217 above the bifurcation of the first diagonal, and manoeuvred back into position. Before wound
218 closure, a drain was inserted to assist with removal of air and fluid from the thorax. Once
219 removed, the wound was then closed in three layers. Sham animals underwent identical

surgery with pericardial rupture although the suture placed through the myocardium was not tightened to cause infarction. Animals were recovered with intraperitoneal atipamezole 0.1 mg/kg (Orion Pharma, Espoo, Finland) and extubated once spontaneous ventilation was established, housed at 30°C for 24 hours and given sterile sodium chloride 0.9% 0.01 mL/g fluid therapy subcutaneously. After 24 hours, normal housing conditions were resumed.

Myocardial Infarction Imaging

Three weeks post-operatively, rats first underwent DEMRI scanning, under Isoflurane anaesthesia as described above. Scout images were taken to confirm correct positioning and to orientate 9x2 mm axial slices from the aortic valve annulus to the apex, perpendicular to the interventricular septum. Cine images were then acquired in long and short-axis views as outlined above. Standard DEMRI inversion recovery was performed using gadolinium complex (gadobendate dimeglumine, Bracco S.p.A, Milan, Italy) with 0.5 mmol/kg administered intravenously via slow injection into the tail vein over 1-2 min. Standard inversion recovery prepared imaging with myocardial nulling was performed 10 min following injection (inversion recovery gradient echo, TI=2.3x R-R [typical R-R 150-200ms], TR≈500 ms; TE=1.6 ms; flip angle=90°; FOV=50×50mm^2; matrix=128×128). Due to information gained from the healthy animal data with the manganese contrast agent experiments (see Results section) as well as technical considerations (i.e. pulse sequence duration), DEMRI T_1 mapping was performed at 20 min following contrast injection upon completion of inversion recovery, at the maximal infarct slice as defined by the cine images (MoLLI: TR>4.5 sec; TE=1.7 ms; flip angle=10°; matrix=128x128; ETL=8; FOV=50x50; 20 time points; trigger delay=1xR-R; slice thickness=2mm;

8 signal averages). Animals were allowed to recover following the scan.

MEMRI was performed 48 h after the DEMRI protocol. Scout images were taken followed by a single short axis cine at the maximal infarct slice, using the method outlined above. MEMRI T_1 mapping was then achieved using one of two manganese-based MRI contrast media being developed for clinical use; EVP1001-1 (n=8) and mangafodipir (n=9) at doses of 22 and 44 µmol/kg respectively, administered via slow intravenous injection into the tail vein over 1-2 min. T_1 mapping was performed at the maximal infarct slice (EVP1001-1, 20 min post injection; mangafodipir 40 min post-injection) defined by the DEMRI scan acquired in the first imaging session. The doses selected of EVP1001-1 and mangafodipir and the timings of the T_1 mapping sequences post-manganese contrast agent administration were informed by the healthy animal data to ensure similar degrees of T_1 enhancement and therefore sensitivity to detect myocardial viability. Finally, a cine acquisition in the short axis at the maximal infarct slice was repeated following contrast injection. Animals were allowed to recover following the scan.

At 12 weeks post-surgery, DEMRI and MEMRI (48 h apart) were repeated using the identical protocols described above. Animals received the same manganese contrast agent at both time points. MRI parameters were unchanged with the exception of an increased FOV (55x55mm) on account of growth of the animals. After the second MEMRI scan, animals were culled by exsanguination by femoral puncture under anaesthesia for tissue harvest. Figure 1. displays a flow chart summarising the imaging of the myocardial infarction cohort.

Figure 1. Myocardial infarction experimental protocol

Flow-chart detailing timing of surgery and imaging with different contrast agents.

Pathology

Hearts were fixed by immersion in 4% paraformaldehyde for 24 hours before being transferred to 70% ethanol and processed to paraffin wax for sectioning thereafter. Serial 5 µm sections were taken at intervals in the short axis from apex to base, corresponding to MRI short axis T_1 mapping data. Staining was performed with Masson's trichrome (MTC) to delineate areas of collagenous fibrosis, staining infarct blue and non-infarct purple, before mounting for computer-aided analysis. Slides were scanned at 20x magnification on a Zeiss Axioscan Z1 (Carl Zeiss AG, Oberkochen, Germany) with infarct size calculated as a percentage of total left ventricular area at the comparable maximal infarct slice defined by MRI. Automated tissue detection was conducted using Tissue Studio v2.4 (Definiens AG, Munich, Germany) as follows: A training set of 4 images was automatically segmented and segments within three 50x50µm regions comprising remote myocardium, infarct, and cross-over regions from each training image (12 regions in total) were manually classified as "Normal myocardium", "Collagen", and "non-tissue". These manual classification samples were used to train the software's automated classification algorithm which was then applied to all images in the dataset. Automated detection produced pixel counts and areas for the ROIs within the left ventricle, from which percentage infarct area at maximal infarct slice was calculated.

Image Analysis

Quantitative analysis of manganese accumulation was achieved by calculation of regional T_1 relaxation times before and after administration of manganese contrast media. The 14-20 images at unique inversion times were exported offline and combined to generate T_1 maps using commercially available software (CVI$^{4.2®}$, Circle Cardiovascular Imaging, Calgary, Canada) using three-parameter non-linear curve fitting as previously described [26].

During the lengthy *in vivo* experiments it was noted that there was an approximate ±10% variation in the measurement of T_1 values in healthy myocardium and skeletal muscle before the administration of manganese contrast agents. In an attempt to compensate for these fluctuations in T_1 measurements myocardial T_1 values were normalized to the T_1 of skeletal muscle, where it was noted that the T_1 values did not vary over the time course of the contrast agent infusion (as detailed in section S1 of the supplemental material). Final normalized T_1 maps were then generated in Matlab (MathWorks Inc., USA) with normalized myocardial T_1 values obtained from regions of interest (ROIs) drawn on the left ventricle. Change in T_1 between the baseline myocardial T_1 and the myocardial T_1 at each time point was then calculated (ΔT_1). Infarct was defined as 2 x standard deviations of infarcted and remote myocardial T_1 with an averaged intermediate value representing borderzone myocardium.

Statistical Analysis

Data are presented as mean ± standard deviation unless otherwise stated. Comparison of the time course curves between the saline (control) and diltiazem-infused groups for each of the 22 µmol/kg $MnCl_2$, 22 µmol/kg EVP1001-1, and 44 µmol/kg mangafodipir was performed using a

two-way analysis of variance (ANOVA) with post-hoc multiple comparison Bonferroni tests for individual time points (compared to baseline). For infarct quantification, DEMRI inversion recovery, DEMRI T_1 mapping, MEMRI T_1 mapping and MTC assessments were compared using Wilcoxon signed rank test (2-tailed), with post-hoc multiple comparison Bonferroni testing. Bland-Altman plots were used to assess agreement between DEMRI inversion recovery, DEMRI T_1 mapping, MEMRI T_1 mapping and MTC, and Spearman correlation tested for relationship between changes in remote T_1 between early and late time points (ΔT_1, dependent variable) and ejection fraction (independent variable). Statistical analysis was performed using GraphPad PRISM (v.7.0, GraphPad Software Inc., La Jolla, CA, USA). Statistical significance was taken as two-sided $P<0.05$.

Results

Comparative and Calcium Channel Dependency of MEMRI Contrast Media

Thirty-one animals underwent experiments with the manganese contrast agent with a concurrent infusion of either 0.9% saline or diltiazem at a median age of 84±19 days, with a median weight of 356±45 g. Four animals (two control and two with diltiazem) were excluded as venous access was compromised resulting in unpredictable contrast agent administration. $MnCl_2$, EVP1001-1 and mangafodipir altered T_1 relaxivity values evident with T_1 mapping (Figure 2A). Mean shortening of myocardial T_1 values was greater with EVP1001-1 as compared to similar concentrations of mangafodipir but commensurate to those observed with $MnCl_2$ (Figure 2B). Peak changes in T_1 values were obtained by 20 min with EVP1001-1 and $MnCl_2$, with persistent T_1 shortening at 60 min. The magnitude of T_1 shortening was dose-dependent for mangafodipir with little change between 40 and 60 min time points (Figure 2B). Overall T_1 shortening with $MnCl_2$, EVP1001-1 and mangafodipir (all 22 µmol/kg) at 20 min were 29.4±5.1%, 28.0±4.4% and 8.5±4.2% respectively. To improve the degree of T_1 shortening with mangafodipir the dosage of manganese administered was increased to 44 µmol/kg resulting in a T_1 shortening of 12.8±3.4% at 20 min which increased to 15.0±2.9% at 40 min. $MnCl_2$ and EVP1001-1 values at 40 min post-administration were unchanged.

Figure 2. T_1 shortening of manganese contrast media over time.

A. Normalized T_1 maps acquired subsequent to infusion of $MnCl_2$, EVP1001-1 and mangafodipir at 20 minute

intervals up to 60 minutes, with associated gradient echo cine images in end-diastole and end-systole. $MnCl_2$ (22 µmol/kg), EVP1001-1 (22 µmol/kg) or mangafodipir (44 µmol/kg) was administered intravenously to isoflurane-anaesthetised healthy rats over 3-4 minutes. Rats were simultaneously administered an infusion of 8 mL/kg 0.9% saline over 3-4 minutes. Note the superior degree of T_1 shortening with $MnCl_2$, and EVP1001-1 at half the molar dosage of manganese as compared to mangafodipir (T_1 reduction of 421.3ms and 357.9ms from baseline with $MnCl_2$ and EVP1001-1 compared to 222.7ms with mangafodipir). **B.** Reduction in mean left ventricular T_1 values over 60 minutes with EVP1001-1 and mangafodipir. $MnCl_2$ (22 µmol/kg; blue), EVP1001-1 (22 µmol/kg; red), mangafodipir (22 [green] or 44 [purple] µmol/kg) was administered to rats (n=4 per group) over 3-4 min. Error bars represent standard deviations from time-points where measurements were recorded (n=4 at each time point). Two-way ANOVA confirmed a dependence of mean myocardial T_1 shortening between each of the contrast agents ($p<0.0001$).

Pre-treatment with a calcium-channel antagonist (diltiazem) inhibited MEMRI induced T_1 shortening (Table 1). This inhibition was similar between all agents with $MnCl_2$ experiencing a maximal mean reduction of 30% in T_1 shortening between the 5 and 60 min time points, while EVP1001-1 and mangafodpir experienced reductions of up to 43% and 32% respectively. There were significant differences between the degree of myocardial T_1 shortening due to each manganese contrast agent in the presence of diltiazem as measured by two-way ANOVA ($MnCl_2$ P=0.0004, EVP1001-1 P<0.0001 and mangafodipir P=0.044).

	Baseline T_1	**Mean T_1 shortening (ms)**			
Time Point (minutes)	**(ms)**	5	20	40	60
$MnCl_2$ + Saline	1286±42	366±72	382±72	389±67	351±35

$MnCl_2$ + Diltiazem (n=3)	1180±93	290±52	288±71	276±56	274±63
EVP1001-1 + Saline	1265±94	354±91	397±73	352±54	383±87
EVP1001-1 + Diltiazem	1375±38	209±27*	225±57*	211±49	226±40*
Mangafodipir + Saline	1366±94	142±52	178±59	206±53	220±61
Mangafodipir + Diltiazem	1219±69	96±34	128±42	158±66	177±24

Table 1. Effect of diltiazem on manganese-induced T_1 shortening. Healthy rats (group sizes n=4 unless otherwise stated) administered $MnCl_2$ (22 µmol/kg), EVP1001-1 (22 µmol/kg) or mangafodipir (44 µmol/kg) over 3-4 min with simultaneous administration of 0.9% saline or diltiazem (100-200 µmol/kg/min) infusion. Note the approximate 30% reduction in mean myocardial T_1 values at each time point, but that there is greater discrimination between the diltiazem and saline infused rats due to the superior T_1 shortening with EVP1001-1. Post-hoc Bonferroni multiple comparisons (manganese agent + saline, manganese agent + diltiazem), significance $p<0.05$ at each time point as compared to saline control indicated by *.

Left Ventricular Function

All contrast agents were well-tolerated across all animal groups. Following administration of gadolinium and EVP1001-1, a transient increase in respiratory rate was observed which was self-limiting and resolved within 1 minute. This was seen in healthy animals, shams and infarcted animals. The present study was not designed to assess safety and tolerability and no further adverse effects were observed.

Healthy myocardial left ventricular ejection fraction was measured before and after manganese contrast agent administration to assess for discernible myocardial depression. The mean LVEF

(± standard deviation) and the mean difference in LVEF at each time point from the cohorts of healthy rats administered with each of the manganese contrast agents with concurrent diltiazem (or 0.9% saline control) infusion are shown in Table 2.

	Mean LVEF (%) and Mean difference in LVEF (%)			
Time point (minutes)	**Baseline**	**15**	**30**	**50**
$MnCl_2$ + Saline	67.5±5.6	73.4±6.3	72.4±5.6	71.1±2.0
Mean Difference (vs. Baseline)	-	5.8±1.3*	4.9±2.6	3.6±5.1*
$MnCl_2$ + Diltiazem	65.0±8.0	58.2±3.9	58.3±6.8	60.1±12.5
Mean Difference (vs. Baseline)	-	-3.2±0.6*	-3.1±2.6*	-4.8±5.1*
EVP1001-1 + Saline	65.3±0.7	71.0±5.5	67.1±2.5	67.1±3.8
Mean Difference (vs. Baseline)	-	4.0±6.8	-0.3±2.6	0.7±5.5
EVP1001-1 + Diltiazem	69.6±6.4	68.3±8.4	63.4±7.3	65.6±7.8
Mean Difference (vs. Baseline)	-	-0.5±3.7	-4.1±4.0	-4.1±3.7
Mangafodipir 22 µmol/kg	63.2±6.3	66.9 (n=2)	65.8±8.2	67.1±7.4
Mean Difference (vs. Baseline)	-	6.9 (n=2)	4.7±7.1	2.5±4.5
Mangafodipir 44 µmol/kg + Saline	73.5±3.1	76.8 (n=2)	76.1±4.9	77.0±4.1
Mean Difference (vs. Baseline)	-	3.9 (n=2)	4.5±1.1	4.6±3.6
Mangafodipir 44 µmol/kg + Diltiazem	64.1±4.8	64.0±8.1	62.5±7.3	62.0±9.5
Mean Difference (vs. Baseline)	-	0.81±6.53	-0.2±6.9	-1.0±4.7

Table 2. Mean LVEF and mean difference in LVEF vs. baseline for the cohort of healthy rats administered

$MnCl_2$, EVP1001-1 and mangafodipir. Group sizes n=3 for EVP1001-1 and mangafodipir 22 µmol/kg and n=4 for $MnCl_2$ and mangafodipir 44 µmol/kg. Calculation of mean only from those time points with n=2 measurements available. Post-hoc Bonferroni multiple comparisons significance P<0.05 at each time point as compared to saline control indicated by *.

No difference was noted between the mean difference in LVEF between baseline and the time points between mangafodipir 22 µmol/kg and mangafodipir 44 µmol/kg (P=0.78) by two-way ANOVA indicating that the higher mangafodipir dosage was well tolerated. There were significant differences detected between the mean difference in LVEF with each of the contrast agents in the presence of diltiazem: $MnCl_2$ 22 µmol/kg (p<0.001 and significant [p<0.05] at the 15 min and 50 min time points on multiple comparison); EVP1001-1 (p=0.02) and mangafodipir 44 µmol/kg (p=0.04). This may potentially indicate that in the presence of depressed LVEF the manganese contrast agents at the dosages used may further compromise myocardial contractility, however further study would be required beyond this pre-clinical study with low numbers of animals. There was no change in left ventricular ejection fraction following manganese administration with any of the agents in the saline control animals. This data supports the assertion that there is minimal change in left ventricular myocardial function in healthy animals with the dosages used in this study. In the infarct group, left ventricular function was assessed at the maximal infarct slice by fractional area change (single slice only due to concern over prolonged anaesthetic risk) before and after manganese-based contrast media administration to assess for discernible myocardial depression. There was no change in fractional area change at this slice following administration of either EVP1001-1 or mangafodipir at early (0.1±1.5%, P=0.82 and 0.1±2.2%, P=0.88) or late (0.2±1.2%, P=0.74 and

0.3±1.8%, P=0.70) imaging time points respectively.

Effect of T_1 Relaxivity of Contrast Media

On comparison of the effect of different contrast agents on T_1 relaxivity, the difference in mean T_1 between infarcted and remote myocardium was highly significant across all contrast agents as well as native T_1 mapping. Remote myocardial mean T_1 was similar between both mangafodipir and EVP1001-1 (Figure 3).

Figure 3. Comparison of the effects of contrast agents on T_1 relaxivity.

Mean T_1 is significantly different between remote and infarcted areas of myocardium for all agents and native T_1 mapping (all P<0.0001). Remote myocardial T1 between manganese contrast agents was comparable (P=0.064).

Viability Assessment Post-myocardial Infarction

Eighteen animals underwent successful surgery (14 permanent coronary artery ligation surgery, 4 sham surgery) at a median age of 58±7 days, with a median weight of 262±52 g. One animal in the surgical cohort died unexpectedly 11 days after surgery, resulting in 17 animals completing the experimental imaging protocol. A further animal died unexpectedly 31 days after surgery, with large myocardial infarction a likely substrate for ventricular arrhythmia as previously observed [28], and 2 animals failed to recover from MRI at the late time point.

Three weeks following surgery, all animals in the infarct cohort had left ventricular impairment

with anterior wall akinesis and wall thinning associated with a reduced left ventricular ejection fraction (42.2±8.1 versus 68.9±9.4% in sham animals, P<0.001). Myocardial infarction was also associated with higher left ventricular end diastolic volume (1.0±0.2 versus 0.7±0.1 mL, P=0.02) and mass (0.7±0.1 versus 0.6±0.04 g, P=0.03). There were no differences in left ventricular function nor volume between animals administered mangafodipir or EVP1001-1 (left ventricular ejection fraction, 41.49±9.86 and 43.10±6.44% respectively, P=0.76; left ventricular end diastolic volume, 1.02±0.21 versus 0.83±0.07 mL respectively, P=0.18).

After surgery, infarct size at the maximal infarct slice was smaller when assessed by MEMRI than DEMRI at 3 weeks (17.4±8% versus 28.5±13%, P<0.05), although the differences were less marked by 12 weeks (20.4±9% versus 28.6±8%, P=0.067, Figure 4).

Figure 4. Comparison of DEMRI and MEMRI infarct quantification by T_1 mapping.

Mean infarct size as a percentage of left ventricular myocardium at maximal infarct short-axis slice in rats with DEMRI and MEMRI T_1 mapping at two time points; early post-MI (3 weeks, n=13, left panel) and late post-MI (12 weeks, n=12, right panel). Infarct size as assessed by MEMRI T_1 mapping is significantly lower than DEMRI T_1 mapping at 3 weeks (P<0.05), a result which is attenuated at 12 weeks (P=0.067). Error bars represent standard deviation. Example T_1 maps with delayed-enhancement and gradient echo cine images are shown for one animal.

At 12 weeks, DEMRI inversion recovery, DEMRI T_1 mapping and MEMRI T_1 mapping of infarct size all correlated independently with histologically quantified infarct size by MTC (all P<0.05). However, unlike manganese, gadolinium-based assessments tended to over-estimate infarct

size by around 10% (DEMRI inversion recovery, bias 11.36%, 95% confidence intervals -9.11 to 31.82, P=0.002; DEMRI T_1 mapping, bias 8.25 %, 95% confidence intervals -10.7 to 27.2, P=0.008; MEMRI T_1 mapping, bias 1.14 %, 95% confidence intervals -14.8 to 17.1, P=0.735; with post-hoc Bonferroni multiple comparisons for P<0.05, Figure 5 A and B).

Figure 5. DEMRI vs MEMRI vs MTC

A. Comparison of magnetic resonance imaging and histological quantification of infarct size. Infarct size as a percentage of left ventricular myocardium at maximal infarct short-axis slice by DEMRI and MEMRI T_1 mapping and histologically with MTC. Note the inverted T_1 colour map configuration between DEMRI T_1 mapping and MEMRI T_1 mapping, calibrated to define infarct (pink) and remote (green) myocardium with intermediate values (yellow). **B**. Bland-Altman plots showing differences between DEMRI inversion recovery (i), DEMRI T_1 mapping (ii) and MEMRI T_1 mapping (iii) for each rat heart. The average difference (bias) between the measurements is shown (dashed lines) ±2xSD (dotted lines) for all three modalities.

There was an inverse correlation between ejection fraction and remote myocardial ΔT_1 (r=-0.61, P=0.022; Figure 6) with greater reduction in remote myocardial T_1 at 3 months with increasing severity of left ventricular impairment. Myocardium remote from the site of infarction (mean T_1 of non-infarcted myocardium) appeared to have lower mean MEMRI T_1 mapping values at late (12 week) compared to early (3 week) time points in animals with the largest infarcts by ejection fraction but this was not statistically significant (mean ΔT_1 -8.39±0.66%, P=0.4, n=3; sham animals with preserved left ventricular ejection fraction mean ΔT_1 7.19±5.93%, P=0.7, n=3).

Figure 6. ΔT_1 in remote myocardium over time vs ejection fraction

Correlation of change in remote myocardial T_1 relaxivity between early and late time points with ejection fraction at 12 weeks post-surgery. There is a significant correlation between ejection fraction and T_1 reduction between early (3 week) and late (12 week) time points (r = 0.61, P = 0.022). Standard error of the mean shown as dashed black line.

Discussion

The present study applies myocardial T_1 mapping to manganese-enhanced MRI, in healthy myocardium in addition to remote myocardium post-infarction. This novel combination of imaging techniques has been employed to directly compare two distinct manganese contrast agents with conventional DEMRI in the assessment of viability by infarct size, as well as examine altered calcium handling in remodelling myocardium over time, building on previous pilot data in myocardial infarction. This work was designed as a precursor to clinical translation of intra-myocardial contrast imaging, for development of this promising field within cardiac MRI which has potential to improve accuracy of myocardial viability assessment, improve understanding of pathophysiology and monitor response to therapy in different forms of cardiomyopathy.

We have demonstrated that MEMRI causes an ionic, concentration and calcium-channel dependent shortening of myocardial T_1 values. We have further shown that MEMRI T_1 mapping provides a better estimate of infarct size than DEMRI using both inversion recovery and T_1 mapping, and correlates with left ventricular remodelling within the remote myocardium

following myocardial infarction. We conclude that MEMRI holds major potential for the assessment of myocardial viability, dysfunction and regeneration with wide ranging clinical applicability.

Chelation, concentration and calcium-channel dependence

Biotransformation of MnDPDP occurs by dephosphorylation and simultaneous transmetallation with zinc facilitating MRI-detectable intracellular manganese uptake, as demonstrated *in vitro* where transmetallation with zinc occurs rapidly, almost to completion, within 1 minute of incubation in human serum [29]. These findings have been reinforced in subsequent animal [30,31] and human studies [32,33]. In the present study, chelated (mangafodipir) and non-chelated ($MnCl_2$ and EVP1001-1) manganese contrast media were compared in healthy myocardium. Intracellular T_1 shortening properties of manganese were clearly demonstrated with a reduction in myocardial T_1 values of 29.4±5.1%, 28.0±4.4% and 12.8±3.4%, compared to baseline values, with 22 µmol/kg $MnCl_2$ and EVP1001-1 at 20 min, and 44 µmol/kg mangafodipir at 40 min respectively. The paramagnetic performance of manganese when administered as EVP1001-1 was highly comparable to that of $MnCl_2$, with rapid increase in T_1 relaxivity over time achieving close to maximal relaxivity by 5 min (Figure 2A). This correlation is expected given intravenous administration of non-chelated manganese ions in both cases. This effect on T_1 shortening was sustained at 60 min and an optimal imaging time point of 20 min was adopted to allow for variation in administration dynamics and utilised in the infarcted myocardium MEMRI experiments. A less marked increase in relaxivity was observed with mangafodipir. This is likely to be due to the need for manganese to become unchelated from

the DPDP ligand as above. To achieve reduction in T_1 comparable to the non-chelated preparations, the dose of mangafodipir was doubled to 44 μmol/kg to obtain similar reductions in myocardial T_1 (Figure. 2B). T_1 relaxivity continued to increase over the measured time period although appeared to begin to plateau from 40 min post-administration. An imaging time point of 40 min was selected as a compromise between practicability and allowing adequate time for unchelation of manganese to achieve sufficient intracellular uptake and therefore provide adequate T_1 shortening. Inhibition of intracellular manganese uptake was evident from a consistently reduced T_1 shortening observed with $MnCl_2$, EVP1001-1 and mangafodipir when co-administered with diltiazem (Table 1). A benzothiazepine, diltiazem binds to L-type calcium-channels at cardiac myocytes and decreases myocardial contractility [13]. Co-administration with manganese-based contrast agents serves to assess manganese uptake in myocardium during calcium channel inhibition. Myocardium in animals pre-treated with diltiazem showed reduction in mean shortening of myocardial T_1 values with both clinical-grade agents, but the magnitude of inhibition was greater with EVP1001-1 compared to similar concentrations of mangafodipir. Due to the superior T_1 shortening with EVP1001-1 at lower doses, there are potentially greater differences between the diltiazem and saline infused animals. These data reinforce the understanding that intracellular manganese uptake is dependent on both L-type voltage-gated calcium-channel as well as sodium/calcium exchanger activity, as previously demonstrated [34].

MEMRI in myocardial infarction

In myocardial infarction, both DEMRI inversion recovery and T_1 mapping consistently

546 overestimated infarct area 3 weeks post-surgery in comparison to MEMRI T_1 mapping. Both
547 DEMRI and MEMRI modalities correlated with histopathological infarct quantification by MTC.
548 However, infarct quantification was similar for histopathology and MEMRI T_1 mapping, whereas
549 DEMRI consistently overestimated infarct size. Contrast imaging of acute myocardial infarction
550 with DEMRI inversion recovery is well-established to overestimate infarct size due to
551 pathologically expanded extracellular space and myocardial oedema. This finding has been
552 observed with preclinical data from swine ischaemia-reperfusion injury indicating discrepancy
553 between DEMRI inversion recovery (both *in vivo* and *ex vivo*) and histological infarct size at 6 h,
554 resolving at 7 days [35]. In clinical studies, imaging too early after infarction results in
555 enhancement of salvaged as well as infarcted myocardium [7] and some degree of myocardial
556 oedema remains and is stable for 7 days following myocardial infarction, reducing at 14 days
557 and near-normalising at 6 months [36]. MEMRI mechanistically circumvents the uncertainty of
558 myocardial oedema as it acts as a specific intracellular agent tracking cardiomyocytes with
559 functional calcium-handling. Furthermore, permanent arterial occlusion models, as used in the
560 present surgical protocol, result in substantially less myocardial interstitial oedema than
561 ischaemia-reperfusion models, even at 24 h [37]. In the present study, gross myocardial
562 oedema is therefore unlikely to persist at 3 weeks, implicating other factors to account for
563 overestimation of infarct size by DEMRI techniques. We hypothesise that dual enhancement
564 with both gadolinium and manganese may occur in areas of injured myocardium where there is
565 residual calcium transport functionality. Early clinical work has explored MEMRI T_1 mapping as a
566 technique to define viability in this way using mangafodipir, demonstrating manganese
567 enhancement in myocardium remote to the infarcted region, 3-4 weeks post-infarction [38].

Whilst $MnCl_2$ carried significant risk of adverse events in cardiac patients which prohibits further clinical development, mangafodipir and EVP1001-1, both having established clinical safety and tolerability, are now primed for clinical applications in cardiac imaging. The clinical significance of viable myocardium defined in this way is as yet unknown and underscores the need for robust clinical trials in this field.

Detection of altered calcium-handling with MEMRI

T_1 mapping of remote myocardium was compared over time following myocardial infarction allowing time for left ventricular remodelling. Changes in T_1 values between early and late time points were compared for each animal. Despite variability between animals, an inverse correlation was observed between ejection fraction and change in T_1 value for all animals, including shams. The significance and precise mechanisms underlying this preliminary finding are unconfirmed as there are contradictory data on activity of voltage-gated L-type calcium-channels in heart failure. L-type calcium-channels in remodelling remote myocardium may have a greater propensity to remain open for longer, on account of prolongation of the plateau phase of the cardiac action potential, as compared to uninjured myocardium resulting in greater relative manganese uptake [39]. A study analysing cardiomyocytes from failing human myocardium observed enhancement of single L-type calcium-channel activity compared to non-failing control myocardium, demonstrating both increased availability and open probability [40]. The data from the present study indicate potential for MEMRI to characterise disordered calcium-handling in failing myocardium, but this requires further exploration in clinical translational studies.

Challenges of the preclinical model

The present study has been designed as a proof-of-concept study of MEMRI in ischaemic cardiomyopathy to inform clinical translation of this imaging modality in ischaemic heart disease as well as other forms of cardiomyopathy. The aim was to assess the application of manganese in non-infarcted myocardium undergoing remodelling following infarction rather than specific dynamics of the infarct region acutely after infarction. Therefore, a permanent coronary artery ligation model was used over an ischaemia-reperfusion model. There are several aspects that are specific to preclinical MR imaging of rodents which do not apply to clinical imaging which are relevant. Despite the excellent spatial resolution of the T_1 mapping sequence used in this study, the small myocardial volume in conjunction with heart rates in excess of 350 beats per minute, obligate free-breathing acquisition with respiratory rates in excess of 40 breaths per minute (with a high degree of variability in both these parameters) provide significant challenges resulting in prolonged T_1 mapping sequences (11-13 minutes) and the margin for error is consequently much narrower than in a clinical equivalent. This constraint resulted in the practical requirement to select one slice representative of the myocardial infarction, rather than acquire a full T1 map short-axis stack. The potential for sampling error between scans was minimized by careful adjudication by two experienced operators at the time of scanning to agree adequate orientation and replicate slice planning methodology, as described above. Whilst unable to guarantee maximal infarct slice, this ensures equivalent slice comparison between scans.

612 The use of T_1 mapping removes the issue of timing in correct nulling, which is made problematic
613 due to the animal-specific issues above. In the imaging of an infarct, it is possible to select an
614 inversion time following manganese administration whereby the infarct is nulled and the
615 myocardium enhances, in an opposite fashion to DEMRI with gadolinium. Inversion recovery
616 imaging with these nulling techniques is easily degraded by artefact of irregular heart rate or
617 breathing and accurate conclusions are highly dependent on the quality of the nulling.
618 Moreover, where more diffuse myocardial processes are concerned, such as remote
619 remodelling in ischaemic cardiomyopathy, T_1 mapping offers the ability to quantify the
620 graduation of T_1 across all regions of myocardium. Finally, in clinical MR imaging, a motion
621 correction algorithm is applied to the T_1 mapping sequence. This algorithm is not available to us
622 in the preclinical setting. These technical factors, unique to the preclinical nature of this study,
623 necessitated the use of normalization of T_1 values and underscore the need for clinical
624 translation.
625
626 **Clinical perspectives**
627 What future diagnostic and therapeutic possibilities does manganese-enhanced cardiac MRI
628 offer the clinician? The prospect of intracellular myocardial tissue characterisation is novel and
629 has far reaching potential. The present study demonstrates unique description of myocardial
630 infarction and viability through calcium-handling which has exciting applications in
631 development of novel therapies in myocardial infarction, recently explored in preclinical
632 ischaemia-reperfusion assessing myocardial regeneration with stem-cell therapy [21] and
633 clinical work establishing safety and tolerability of chelated manganese contrast agents in

ischaemic heart disease[38]. Beyond myocardial infarction, we have demonstrated potential to detect altered calcium-handling non-invasively and scope for earlier detection and quantification of cardiomyopathy. The present preclinical study highlights the need for clinical translation of these agents, where image acquisition is vastly superior, in a patient population which accurately represents the substrate for disease underlying the pathology.

Conclusion

The present study demonstrates the utility of MEMRI with two distinct agents, chelated and non-chelated manganese, as a non-invasive imaging modality which can accurately quantify viable myocardium. Furthermore, these data indicate an ability to detect and quantify altered calcium-handling in the remodelling remote myocardium. This novel technique has potential to actively quantify viable myocardium, rather than inferring viability using infarct extent as a surrogate. Furthermore, calcium handling dysfunction observed in a wide range of cardiomyopathies and heart failure syndromes eludes current non-invasive investigation; an application where MEMRI holds great promise. Clinical translation of the work presented here is an essential next step.

Abbreviations

DEMRI: delayed-enhancement magnetic resonance imaging

DPDP: dipyridoxyl diphosphate

ETL: echo train length

FOV: field of view

MEMRI: manganese-enhanced magnetic resonance imaging

MoLLI: modified Look-Locker inversion recovery

MRI: magnetic resonance imaging

MTC: Masson's trichrome

NMR: nuclear magnetic resonance

ROI: region of interest

TE: echo time

TR: repetition time

Declarations

Ethics approval

Ethical approval was granted for all animal studies by the University of Edinburgh Animal Welfare and Ethical Review Body.

Consent for publication

Not applicable.

Availability of data and material

The datasets generated and/or analysed during the current study are not publicly available due to commercial regulations pertaining to involved third parties, but may be available from the corresponding author on reasonable request.

Competing interests

RLJ is currently employed by GlaxoSmithKline, which contributed to funding for the study through a fellowship awarded to DML. Neither the funding agency nor any outside organization has participated in study design or have any competing interests. GlaxoSmithKline approved the final manuscript.

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Authors' contributions

NBS and DML acquired, analysed and interpreted the data and compiled the original manuscript. NBS and LLP performed the surgical protocol and contributed to critical revision of the manuscript. MAJ, RJL, DML and NBS undertook imaging protocols and contributed to critical revision of the manuscript. DML, NBS, SIS, MAJ, DEN, GAG, MRD and PCY were involved in study design, supervised the protocols and contributed to critical revision of the manuscript. NBS and GP undertook inter-operator variability studies and analysis. SIS, MAJ and PCY were joint senior supervisors on the project. RLJ supervised DML through the GlaxoSmithKline fellowship, contributed scientifically to this work, and contributed to the critical revision of the manuscript. PCY provided EVP1001-1 and imaging expertise.

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