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Imaging assessment of cardioprotection mediated by a dodecafluoropentane oxygen-carrier administered during myocardial infarction

Zhonglin Liu^{a,*}, Christy Barber^a, Akash Gupta^b, Li Wan^a, Young-Wook Won^c, Lars R. Furenlid^a, Qin Chen^d, Ankit A. Desai^b, Ming Zhao^e, David A. Bull^c, Evan C. Unger^{a,f}, Diego R. Martin^{a,*}

^aDepartment of Medical Imaging, University of Arizona, Tucson, AZ, United States of America

^bDivision of Cardiology of Department of Medicine, University of Arizona, Tucson, AZ, United States of America

^cDivision of Cardiothoracic Surgery of Department of Surgery, University of Arizona, Tucson, AZ, United States of America

^dDepartment of Pharmacology, University of Arizona, Tucson, AZ, United States of America

^eFeinberg School of Medicine, Northwestern University, Chicago, IL, United States of America

^fNuvOx Pharma, LLC., Tucson, AZ, United States of America

Abstract

Introduction: The objective of this study was to investigate the cardioprotective effects of a dodecafluoropentane (DDFP)-based perfluorocarbon emulsion (DDFPe) as an artificial carrier for oxygen delivery to ischemic myocardium, using ^{99m}Tc-duramycin SPECT imaging.

Methods: Rat hearts with Ischemia-reperfusion (I/R) was prepared by coronary ligation for 45-min followed by reperfusion. The feasibility of ^{99m}Tc-duramycin in detecting myocardial I/R injury and its kinetic profile were first verified in the ischemic hearts with 2-h reperfusion ($n = 6$). DDFPe (0.6 mL/kg) was intravenously administered at 10 min after coronary ligation in fifteen rats and saline was given in thirteen rats as controls. ^{99m}Tc-duramycin SPECT images were acquired in the DDFPe-treated hearts and saline controls at 2-h (DDFPe-2 h, $n = 7$ and Saline-2 h, $n = 6$) or 24-h (DDFPe-24 h, $n = 8$ and Saline-24 h, $n = 7$) of reperfusion.

Results: SPECT images, showing “hot-spot” ^{99m}Tc-duramycin uptake in the ischemic myocardium, exhibited significantly lower radioactive retention and smaller hot-spot size in the DDFPe-2 h and DDFPe-24 h hearts compared to controls. The infarcts in the Saline-24 h hearts extended significantly relative to measurements in the Saline-2 h. The extension of infarct size did not reach a statistical difference between the DDFPe-2 h and DDFPe-24 h hearts. *Ex vivo* measurement of ^{99m}Tc-duramycin activity (%ID/g) was lower in the ischemic area of DDFPe-2 h and DDFPe-24 h than that of the Saline-2 h and Saline-24 h hearts ($P < 0.05$). The area of injured

*Corresponding author at: P.O. Box 245067, Tucson, AZ 85724-5067. zliu@email.arizona.edu (Z. Liu), dmartin@radiology.arizona.edu (D.R. Martin).

myocardium, delineated by the uptake of ^{99m}Tc -duramycin, extended more substantially outside the infarct zone in the controls.

Conclusions: Significant reduction in myocardial I/R injury, as assessed by ^{99m}Tc -duramycin cell death imaging and histopathological analysis, was induced by DDFPe treatment after acute myocardial ischemia. ^{99m}Tc -duramycin imaging can reveal myocardial cell death in ischemic hearts and may provide a tool for the non-invasive assessment of cardioprotective interventions.

Keywords

Myocardial ischemia-reperfusion; Dodecafluoropentane perfluorocarbon emulsion; Phosphatidylethanolamine; ^{99m}Tc -Duramycin; Cell death imaging

1. Introduction

Myocardial infarction (MI) is the leading cause of death in the United States. Typically, myocardial damage begins within 20 to 30 min of the onset of acute ischemia, with irreversible myocyte necrosis occurring over the course of several hours. Prompt revascularization therapy to restore oxygenated blood flow is regarded as the primary means of limiting heart damage after acute MI. The best clinical outcomes can be achieved when reperfusion is initiated within 2 h after the onset of symptoms. However, most patients often receive intervention outside of the optimal 2-h window. After a critical period of coronary occlusion, the injured myocardium becomes unsalvageable [1-3]. Thus, a cardioprotective strategy that extends the time window until revascularization therapy can be applied is needed urgently. This study was designed to investigate the potential of using dodecafluoropentane emulsions (DDFPe) as a cardiac protective agent in the presence of reperfusion to enhance oxygen content to ischemic but viable myocardium.

As a nanodroplet (250–300 nm) form of perfluorocarbon emulsion, DDFPe can transition to the gaseous state at 29°C and exists as a gas at body temperature [4]. Due to intravascular pressure, the nanodroplets expand only slightly and do not shift to microbubble forms in the body at 37°C. The short half-life of DDFPe in the blood (1.8 to 2.5 min) results in complete removal of typical doses through respiration by 2 h. In the setting of arterial vascular occlusions, these nanodroplets are presumably small enough to reach ischemic tissues, beyond an erythrocyte-blocking thrombotic occlusion, through intra-thrombus fissures and collateral microvasculature. The nanodroplets may also deliver oxygen to ischemic tissues by simple diffusion gradients [4-7]. Such properties allow DDFPe to deliver many times the oxygen content of liquid perfluorocarbon and can compensate for up to 90% of the oxygen deficiency. Significant reduction of cerebral infarct size in rat and rabbit stroke models has been achieved by single or repeated doses of DDFPe [4-6,8-14]. In a study of a mouse model with MI, caused by irreversible permanent occlusion of the left anterior descending (LAD) coronary artery, DDFPe was administered prior to the coronary occlusion and shown to be able to elicit a cardioprotective effect that persisted well beyond the assumed elimination time of DDFPe [3].

Although DDFPe may offer a simple and effective means to support ischemic but viable myocardium for safe revascularization by transporting oxygen to areas inaccessible to red

blood cells, no direct experimental evidence is yet available to show a cardioprotective effect when it is combined with a reperfusion procedure. There is a concern that excess generation of reactive oxygen species (ROS) by DDFPe-enhanced oxygen delivery may accelerate reperfusion injury via a programmed cell death (apoptosis) mechanism, which is a caspase-dependent process that can be triggered by reperfusion or reoxygenation. Furthermore, the mechanism of the ischemic tissue protection effect of DDFPe remains uncertain. The time course of effectiveness of DDFPe has not been fully investigated. Accordingly, we applied a cutting-edge imaging method, by targeting of apoptotic cells, to assess the utility of DDFPe as artificial carriers for oxygen delivery to ischemic myocardium in rat heart models with 2-h and 24-h reperfusion.

Phosphatidylethanolamine (PE) is the second most abundant mammalian phospholipid and >80% of PE is normally located in the inner leaflet of the cell membrane [15,16]. PE also represents >35% of the phospholipid phosphorus of the inner mitochondrial membrane [17]. Exposure of PE molecules along with phosphatidylserine (PS) in the outer part of the membrane is a primary process during apoptosis [18,19]. Cell-surface PE exposure on apoptotic cells is well correlated with PS exposure in the early phase of apoptosis [20]. A PE specific molecular probe, ^{99m}Tc -labeled duramycin (^{99m}Tc -duramycin), has been developed for detecting apoptotic cell death in preclinical studies during recent years. With only 19 amino acids, duramycin is the smallest known polypeptide that has a 3-D binding site that binds the head group of PE with high affinity and exclusive specificity [21-23]. We have previously demonstrated the feasibility of ^{99m}Tc -duramycin for imaging cerebral ischemia-reperfusion (I/R) injury and we have shown its favorable pharmacokinetics in a rat stroke model [24]. We hypothesized that SPECT imaging with ^{99m}Tc -duramycin could provide insight into molecular processes related to DDFPe cardioprotection in ischemic-reperfused hearts. Because SPECT imaging with ^{99m}Tc -duramycin permits quantitative assessment of the extent of cell injury, we presumed that noninvasively collected in vivo ^{99m}Tc -duramycin data could differentiate reversible myocardial injury from irreversible injury and estimate the effectiveness of DDFPe in cardioprotection.

2. Materials and methods

2.1. DDFP nano-emulsion

A DDFP nano-emulsion, NVX-308, was prepared and provided by NuvOx Pharma, LLC. (Tucson, AZ) for this study. It is composed of emulsified dodecafluoropentane which is a unique fluorocarbon material with quasi-bubble properties [4]. Each vial of the nano-emulsion was prepared and stabilized to maintain the particle size at ~250 nm. For animal experiments in this study, a diluted solution of DDFPe was prepared in saline for intravenous administration at a dose of 0.6 mL/kg.

2.2. ^{99m}Tc -duramycin

Duramycin was conjugated with 8 equivalents of succinimidyl-6-hydrazinonicotinamide acetone hydrazone (HYNIC-NHS). The resulting HYNIC-duramycin was formulated with tricine and trisodium triphenylphosphine-3,3',3''-trisulfonate (TPPTS) as co-ligands [23]. Single-step kit composed of 15 μg HYNIC-duramycin, 30 mg tricine, 10 mg TPPTS, and 15

µg tin chloride dehydrate obtained from Molecular Targeting Technologies, Inc. (MTTI) (West Chester, PA) was labeled with ^{99m}Tc . ^{99m}Tc -pertechnetate (740 MBq) in 500 µL of saline was added to the vial and heated at 80°C for 20 min. Radiolabeled product was purified by reverse phase (RP) HPLC using a C18 column with a gradient elution of 25 mM NaH_2PO_4 at pH 6.7 and acetonitrile at a flow rate of 1 mL/min [25]. Radiochemical purity of ^{99m}Tc -duramycin exceeded 95% for all experimental injections.

2.3. Ischemic-reperfused rat heart model preparation

Male Sprague-Dawley rats (weighing 250–350 g) were anesthetized with 1.0%–1.5% isoflurane. The model of myocardial ischemia-reperfusion was developed using the technique described previously [26]. The rat was ventilated using a volume-controlled Inspira Advanced Safety Ventilator (Harvard Apparatus, Holliston, MA) with oxygen. The chest was opened with a left intercostal thoracotomy incision at the 5th intercostal space. A ligature placed around the left coronary artery (LCA) was pulled tight by passing the suture through a polyethylene tubing and clamping it for coronary occlusion. Regional myocardial reperfusion was elicited by releasing the ligature at 45 min post LCA occlusion. The chest of the rat was closed in layers with 4–0 silk sutures. All animals were weaned from ventilation and isoflurane anesthesia once they resumed spontaneous respiration.

2.4. Validating an association between MI and ^{99m}Tc -duramycin imaging

In order to verify the feasibility of detecting myocardial ischemia-reperfusion injury using ^{99m}Tc -duramycin imaging and obtain its radiopharmaceutical kinetics in the rat heart model, six rats (**Group I**) with 45-min myocardial ischemia and 2-h reperfusion without any treatments were dynamically imaged using FastSPECT II, which is a high-resolution stationary SPECT imager with dynamic imaging capabilities [27]. ^{99m}Tc -duramycin was intravenously administered via a catheter in the tail vein. Dynamic list-mode SPECT projection data were collected for 10 min immediately after tracer injection. Subsequently, 5-min projection data were acquired every 15 min for 2 h post-injection. Sixteen projections were obtained to generate the data set for tomographic reconstruction.

2.5. ^{99m}Tc -duramycin imaging of ischemic-reperfused rat hearts treated with DDFPe

In 15 rats with LCA ligation, we administered a single dose (0.6 mL/kg) of 2% DDFPe intravenously at 10 min after LCA ligation. The treatment dose was derived from that used in animal models reported previously [3-6,8-12]. The dose and administration schedules were preliminarily verified in our pilot studies, in which no adverse events, such as animal death or difficulty in recovery from surgery and anesthesia, were encountered. For a control arm, the same volume of saline was administered in the same manner in thirteen other rats with LCA ligation. The DDFPe-treated rats were randomly divided into two groups to receive ^{99m}Tc -duramycin injection at 2-h (**Group II**: DDFPe-2 h, $n = 7$) and 24-h (**Group III**: DDFPe-24 h, $n = 8$) post reperfusion for SPECT imaging. Similarly, the controls received ^{99m}Tc -duramycin for imaging after 2-h (**Group IV**: Saline-2 h, $n = 6$) or 24-h reperfusion (**Group V**: Saline-24 h, $n = 7$). ^{99m}Tc -duramycin (55.5–92.5 MBq) was injected via a tail vein when the rat was anesthetized. Two hours after tracer injection, cardiac static images were acquired for 10 min using FastSPECT II.

2.6. ^{99m}Tc -duramycin image processing

Reconstructions of FastSPECT II data were processed using 30 iterations of the OS-EM algorithm. Tomographic slices with one-voxel thickness (0.5 mm) were produced using AMIDE 1.0.4 software. A lesion was interpreted as positive if focal uptake (hot-spot) localized on the left ventricular (LV) wall at least three adjacent tomographic slices. In dynamic images of Group I, region-of-interest (ROI) analysis was applied to quantify the radioactive distribution of the cardiac blood pool, the LV wall, and the liver. 3D Freehand ROIs were manually established over normal myocardial zones supplied by the right coronary artery (RCA). 3D Isocontour ROIs over the cardiac blood pool, LV hot-spots, and the liver were drawn with a 3D Isocontour ROI establishment on coronal slices based on the 1-min and 120-min images. The 1-min or 120-min ROIs were applied to all the dynamic images for determining average counts per voxel. After correction for acquisition time and radioactive decay, time activity curves (TACs) over the normal zone and hot-spot of LV wall, the cardiac blood pool, and the liver were plotted by normalizing radioactive counts at each time point to counts at 2-min to avoid the variation of injected dose. SigmaPlot 12 software (Systat Software Inc., Chicago, IL) was applied to fit the TACs using a set of kinetic functions with Exponential Decay (Single, 3 Parameter) and Exponential Rise to Max (Single, 3 Parameter).

In Group II-IV, the LV hot-spot volume (mm^3) was estimated from slices of FastSPECT II images of all animals collected at 2 h post-injection by the 3-D Isocontour ROI analysis. The ROIs were defined so that they encompassed all neighboring values above 55 percentage of the maximum threshold value in the myocardium. After acquiring the number of voxels of the hot-spot, mean radioactivity per voxel was estimated and used to calculate the radioactive ratio of hot-spot to cardiac blood pool and remote non-ischemic LV wall, respectively.

2.7. Postmortem analysis

The rat chest was reopened and the LCA was re-occluded while the animal remained ventilated and anesthetized. Evans blue (10%, 0.5 mL) in PBS buffer was injected through the femoral vein, allowing the dye to stain the non-ischemic territory of the heart before the rat was euthanized.

The entire heart, blood, and other organs were harvested, weighed, and measured for radioactivity using a CRC-15 W Dose Calibrator/Well Counter (Capintec, Ramsey, NJ), which is a combination of dose calibrator and well counter. We measured the radioactivity (μCi) of the harvested organs using the dose calibrator when the tissue radioactivity was $\geq 2.0 \mu\text{Ci}$ to calculate the percentage of the injected dose per gram of tissue (%ID/g). When tissue radioactivity was $< 2.0 \mu\text{Ci}$, we measured the tissue using the well counter. Radioactive counts per min (CPM) were converted into μCi using a calibration procedure. The heart was rinsed of excess dye with cold saline solution. The LV was transversely sectioned, typically generating 3 slices, and a section midway between the apex and base was selected for immediate fixation in 10% buffered formalin. The remaining LV sections were cut into five radial slices, photographed using a digital camera, and then incubated in 1% PBS-buffered triphenyltetrazolium chloride (TTC) solution at 37°C for 20 min. TTC-stained LV slices

were mounted on glass slides and laid on phosphate plates at 4°C for 5–10 min of autoradiograph exposure. Digital photographs were taken again of both sides of each LV slice. The LV digital autoradiographs were read with a Fujifilm BAS-5000 reader. The area unstained by Evans blue was identified as the ischemic area at risk (IAR). The TTC-negative area (white or pale) was recognized as infarcted myocardium and quantitatively analyzed using the software of SigmaScan (SPSS Science, Chicago, IL) in trace-measurement mode. The results of IAR and infarct measured on each LV slice were averaged and the infarct size of whole LV was calculated as a percentage of total LV mass (% LV) and then normalized by the size of IAR (% IAR). Upon nonischemic viable (Evans blue and TTC positive) and ischemic nonviable (Evans blue and TTC negative) segments were ascertainably dissected from all tissue slices, the remaining tissue segments were characterized as mixed-viability tissues. The dissected tissue samples were weighed and gamma-counted after radioactive decay for 24–48 h. The ratio of the infarcted and mixed-viability tissue to remote nonischemic viable myocardium was calculated, respectively. The existence of radioactive uptake in the ischemic myocardium on autoradiograph images was used to validate the in vivo focal uptake of ^{99m}Tc -duramycin detected by FastSPECT II imaging.

The LV segment unstained by TTC was formalin-fixed and embedded in paraffin and cut at serial 5- μm cross-sections for hematoxylin and eosin (H&E) staining and immunohistochemical (IHC) staining to evaluate the overall architecture, MI, inflammatory cell infiltration, and cleaved caspase-3 expression [28]. Deparaffinized section were blocked by 5% BSA 2% Goat serum in 1 $\times$ PBS for an hour. Primary antibody with rabbit anti-cleaved caspase-3 monoclonal antibody (Cell Signaling Technology, Beverly, MA) was incubated at 4°C overnight. The primary antibody was detected using an anti-rabbit biotinylated secondary anti-body followed by biotinylated-streptavidin-horseradish peroxidase and 3,3'-diaminobenzidine system. Digital H&E and IHC images were captured using an Olympus microscope.

2.8. Ethics

The animal experiments were performed in accordance with Principles of Laboratory Animal Care from the National Institutes of Health (NIH Publication 85–23, revised 1985) and were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Arizona.

2.9. Data analysis

All quantitative results were expressed as mean $\pm$ S.E.M. Comparisons between two variables were performed with one-way analysis of variance. A probability value <0.05 was considered significant.

3. Results

3.1. Kinetic features of ^{99m}Tc -duramycin uptake in myocardium with ischemia-reperfusion

The feasibility of detecting myocardial ischemia-reperfusion injury by ^{99m}Tc -duramycin was verified in the rat hearts of Group I. Representative dynamic ^{99m}Tc -duramycin images

in the rat heart models are shown in Fig. 1. Focal radiotracer uptake (hot-spot) was visualized starting at 15 min post tracer injection within the LV anterior wall, lateral wall, and apex. The hot-spot was unequivocally localized within 30 min. At 2 h, the radioactive level of the hot-spot was equal to or higher than that of the liver. As a result, hot-spots were detected by FastSPECT II imaging in all hearts of Group I. Fig. 2 shows marked cardiac blood pool and LV remote wall on 1-min image, ischemic wall with hot-spot uptake of ^{99m}Tc -duramycin on 120-min image, and co-registered 1-min and 120-min images to define the association of hot-spot with the heart in a representative rat. ROI analyses for generating TACs are representatively demonstrated in Fig. 2 and resulted TACs are presented in Fig. 3. We normalized the radioactivity at each time point of TACs to the initial activity at 2 min post-injection. ^{99m}Tc -duramycin TACs exhibited a continuous uptake pattern in the LCA ischemic areas relative to the washout prototype in the normal myocardial zones. The difference observed at each point, from 5 min to 2 h, between the LCA hot spot of the ischemic area and the normal zones was significant ($P < 0.05$). The fractional retention of ^{99m}Tc -duramycin expressed as a percentage at 2 h from the hot-spot was significantly greater than that from the remote viable zone in all hearts (356.0 ± 52.4 vs. 20.1 ± 4.8 , $P < 0.001$). The accumulation of ^{99m}Tc -duramycin in the ischemic area at 2 h was increased 3.56-fold relative to initial radioactivity at 2 min. The ratio of the hot-spot to normal myocardial activity at 2 h post-injection was 22.4-fold higher than that at 2 min (14.1 ± 3.2 vs. 0.63 ± 0.06 , $P < 0.001$). Curve fitting results of the TACs of blood, normal zone, and ischemic area in the ischemic-reperfused hearts of Group I are shown in Fig. 4. Kinetics of ^{99m}Tc -duramycin in the blood and healthy remote myocardium exhibited exponential decay patterns at a 3-parameter function. The average half-life ($t_{1/2}$) of the radioactivity was 11.3 min for the blood and 14.6 min for the normal myocardium. In ischemic myocardium, ^{99m}Tc -duramycin was shown with an exponential rise to maximum function. It had a period of rapid increase in radioactive accumulation from 1 to 44 min, followed by a period of slow increase up to 120 min.

3.2. Reduction of ^{99m}Tc -duramycin myocardial uptake induced by DDFPe

In all rats of Group II-V treated with DDFPe or saline, SPECT images acquired at 2 h after ^{99m}Tc -duramycin injection showed hot-spot radioactive uptake in the left ventricular wall like the distribution of ^{99m}Tc -duramycin in I/R hearts of Group I. As representatively presented in Fig. 5, the overall radioactive levels of hot-spots in the rat hearts of Group II and III were lower than what observed in those of Group IV and V. Fig. 6 illustrates the delineation of LV wall, hot-spot radioactive uptake in the ischemic zone detected by FastSPECT II ^{99m}Tc -duramycin imaging, and ROI establishment to quantify the hot-spots. The results of quantitative analysis in the hot-spots of I/R hearts with and without DDFPe treatment are shown in Fig. 7. The measurements of hot-spot volume (mm^3) in the DDFPe-protected hearts were significantly smaller than that in the control hearts (169.0 ± 15.4 vs. 212.9 ± 12.3 at 2-h reperfusion and 239.0 ± 16.9 vs. 293.3 ± 37.5 , $P < 0.01$). Based on the averaged radioactive intensity determined by ROI analysis on reconstructed ^{99m}Tc -duramycin images, the ratio of LV hot-spot to remote healthy myocardium in DDFPe treated hearts was significantly lower than that in the control hearts (5.9 ± 0.4 vs. 9.3 ± 1.4 at 2-h reperfusion and 3.7 ± 0.4 vs. 5.3 ± 0.5 at 24-h reperfusion, $P < 0.01$).

3.3. Ex vivo measurements of ^{99m}Tc -duramycin uptake in the ischemic-reperfused hearts

The results of ^{99m}Tc -duramycin biodistribution (%ID/g) in the major organs are summarized in Table 1. ^{99m}Tc -duramycin showed prominent renal excretion and moderate uptake in the liver. The radioactivity of blood and lungs were low and had no statistical differences among four groups ($P > 0.05$). The results of biodistribution measurements of dissected left ventricles are shown in Fig. 8. Radioactivity in the whole hearts and dissected LV myocardium subjected to 2-h reperfusion was significantly lower in the DDFPe-treated group than that in the Control group (Whole heart: 0.83 ± 0.14 vs. 1.34 ± 0.12 , $P = 0.016$; LV: 0.98 ± 0.17 vs. 1.53 ± 0.14 , $P = 0.029$), respectively. In the hearts exposed to 24-h reperfusion, the radioactive differences between DDFPe-treated and correspondent control hearts were not statistically significant (Whole heart: 0.76 ± 0.09 vs. 0.73 ± 0.05 , $P = 0.783$; LV: 0.87 ± 0.06 vs. 0.94 ± 0.12 , $P = 0.628$). ^{99m}Tc -duramycin uptake in the IAR delineated by Evans blue staining was significantly lower in the DDFPe-treated group with 24-h reperfusion than that in the group received saline 1.53 ± 0.13 vs. 2.19 ± 0.11 , $P = 0.003$). ^{99m}Tc -duramycin activity in the infarcted segments of the hearts defined by negative TTC staining exhibited no statistic difference between Group III and V with 24-h reperfusion ($P > 0.05$). In contrast, the radioactivity in the infarct zones of Group IV received saline was significantly higher than in the infarcted tissues of Group II treated by DDFPe as ^{99m}Tc -duramycin was injected at 2 h post LCA reflow. ^{99m}Tc -duramycin activity ratios of ischemic zones to remote healthy areas in the hearts of Group II-V are summarized in Table 2.

3.4. Autoradiography, IAR, and infarct measurements

Fig. 9 shows representative photographs of autoradiograph images, Evans blue and TTC-stained LV slices, and postmortem measurements summarized in bar graphs. The measurements of IAR (% LV) showed no difference among the hearts with 2-h (40.9 ± 7.8 vs. 42.2 ± 3.5 , $P > 0.05$) and 24-h reperfusion (43.3 ± 2.8 vs. 39.3 ± 2.7 , $P > 0.05$) subjected to DDFPe and saline administration. The infarct sizes (%LV) in DDFPe-treated hearts with 2-h reperfusion were not significantly different compared to the measurements in the control hearts. However, infarct size (% LV) in the DDFPe-treated group with 24-h reperfusion was significantly smaller than that in the matching control group. After normalization by IAR, the infarct sizes (% IAR) in the 2-h reperfused hearts with DDFPe treatment were significantly smaller, relative to the measurements obtained from the corresponding controls (33.8 ± 5.4 vs. 48.5 ± 2.6 , $P < 0.01$). The infarcts increased to a significantly greater degree in control animals, comparing 2-h and 24-h reperfusion results, increasing from 48.5 ± 5.1 to 68.8 ± 4.2 ($P < 0.01$), as compared to the DDFPe treated animals, which demonstrated an averaged increase from 33.8 ± 5.4 to 40.0 ± 4.1 ($P < 0.05$).

3.5. Histopathology and IHC assay

Postmortem histopathology showed morphological evidences of myocardial injury in the LV sections as representatively presented in Fig. 10. Hypereosinophilia of the cytoplasm, revealed by H&E staining, is a characteristic of myocardial ischemia. The presence of necrotic myocardial cellular features, including shrinkage and darkly stained pyknotic nuclei, and an interface of acute inflammation separating the infarct zone from viable myocardium, was observed in all hearts. Neutrophil infiltration was predominantly present

by 24 h at the ischemic injury border areas. Morphological evaluation of H&E stained sections showed extensive apoptosis in the ischemic-reperfused myocardium. High apoptotic activity in the injured myocardium was further observed by cleaved caspase-3 IHC staining as demonstrated in Fig. 11. Nuclear morphology of most of the caspase-3-stained cells was consistent with apoptosis, with signs of cell shrinkage, nuclear fragmentation, and chromatin condensation. There were many brown-stained positive cells of cleaved caspase 3 in the LV sections of the control rats received saline, but lower number of caspase 3 positive cells were observed in the DDFPe treatment groups. No cells with positive caspase-3 staining could be detected in remote regions of the myocardium, outside of the ischemic zone.

4. Discussion

A simple and effective cardioprotective intervention, able to extend the time window until safe revascularization therapy is available, holds great promise in salvaging ischemic reversible myocardium. A variety of experimental oxygen delivery systems developed to improve tissue oxygenation have failed in clinical trials due to either toxicity or impracticality. DDFPe is a promising agent for protection of the ischemic myocardium even though many issues need to be addressed for clinical translation [4,10].

In this study, we evaluated whether a single dose of DDFPe could provide cardioprotection in transiently ischemic-reperfused rat hearts, when the agent was delivered during the acute ischemic period; and we explored the use of ^{99m}Tc -duramycin for in vivo detection of ischemic myocardial injury for this rat model. As a result, significant tolerance to myocardial ischemia-reperfusion injury, as assessed by noninvasive in vivo infarct-avid imaging and ex vivo histological assay, was successfully achieved. Moreover, the effectiveness of DDFPe cardioprotection was found both at early (2–4 h) and later phases (24 h) after coronary flow reconstitution. Our imaging results revealed the capability of ^{99m}Tc -duramycin for effective visualization of myocardial injury and distinction of the intensity of the injury.

In the formulation of DDFPe used in this study, dodecafluoropentane exists as 250–300 nm nanodroplets. Because of intravascular pressure, the nanodroplets in the body at 37°C expand only slightly allowing facilitation of respiratory gas dissolution. Consequently, the measured cardioprotection at the early phase might result from a quick diffusion of the DDFPe nanodroplets through ischemic tissues via a serial transport mechanism, beyond erythrocyte accessibility, as an alternative pathway for oxygenation of the ischemic myocardium [11,12]. It has been characterized that oxygen transportation by DDFPe does not need red blood cell flow due to its transition from liquid to gas phase. At 37°C, DDFPe can absorb 7 times more oxygen than in the liquid phase. In this study, we did not collect blood samples for blood gas analysis or use a device to noninvasively measure tissue oxygen concentration. However, previous studies performed by Lundgren et al. showed that abdominal muscle oxygen tension in rats, given an infusion of 0.5 mL/kg DDFPe exceeded those in control rats by 50–100% for about 2.5 h [14]. In another study conducted Mullah et al., cerebral tissue oxygen tension in rats with traumatic brain injury (TBI) was increased to a peak of 143% at one hour after administration 0.5 mL/kg DDFPe while oxygen levels remained depressed in the control rats [29]. Furthermore, Johnson et al. conducted a study to

investigate the oxygenation ability of DDFPe in the tumor sites of mice with human Hs-766 T pancreatic cancer xenografts. When DDFPe was given at doses of 0.3, 0.45 and 0.6 mL/kg, tumor pO₂ was proportionally increased up to 400% at the highest dose of 0.6 mL/kg [30]. These findings suggest that DDFPe is able to raise oxygen saturation 40–100% and compensate for 90% of the oxygen deficiency [13,14]. Thus, the capacity for DDFPe-mediated oxygen delivery to ischemic tissues may account for the cardioprotective findings shown in our study.

Cardioprotective potential of DDFPe was previously investigated in a mouse heart model with permanent coronary ligation [3]. Whether DDFPe oxygenation could provide a more pronounced protective effect when it is given in the ischemic hearts followed by revascularization has yet to be investigated. The models used in this study are more clinically relevant to a treatment administered during an active ischemic event followed by a revascularization intervention. Unlike the previous study [3], in which the artificial oxygen carrier was given before coronary artery occlusion, the present study was designed to administer the DDFPe after arterial occlusion and prior to reperfusion, better mimicking a clinical scenario. The protective effects of DDFPe on attenuating myocardial ischemia-reperfusion injury was assessed at early (4 h) and late (24 h) phases of reperfusion, respectively, by in vivo imaging and ex vivo assays. Our experimental protocols yielded evidence that DDFPe could be effective in generating oxygenation that led to initial cardioprotection shown mainly in the early reperfusion model and triggering postconditioning-like protection that extended into the late reperfusion. As a result, we found that significant infarct growth in the IAR of control subjects occurred between 2 h and 24 h after reperfusion, but not in the DDFPe treated animals. This finding, which is not testable using a permanent coronary ligation model, indicates that a single dose administration of DDFPe, following a period of ischemia and reperfusion, may elicit cardioprotection beyond any oxygen transport potential of DDFPe and the retention time of the agent. It is observed that the pharmacodynamics of DDFPe results in rapid transformation to a gas phase after injection, followed by rapid clearance through ventilation, with a circulating half-life of 2.2 ± 1.2 min [3,31]. DDFPe systemic elimination was almost complete 2 h after administration [31].

It may be that the DDFPe effects are mediated via a reactive oxygen species (ROS) signaling pathway [3], even though we have not yet performed direct measurements to determine the levels of ROS or assess signaling pathways associated with ROS. In this hypothesis, reintroduction of oxygen after transient ischemia causes the excess generation of ROS, which stimulates the release of cytokines and expression of adhesion molecules on the surface of damaged cells within the re-oxygenated tissue, resulting in various detrimental processes, including myocardial inflammation, and apoptosis [32-39]. On the other hand, low levels of reactive species may act as secondary messengers, referred to “ROS signaling” in ischemic preconditioning, modulating cardioprotective signaling pathways by covalent modification of target molecules, such as activation of the ATP-sensitive potassium channel (mitoK_{ATP}) [40,41]. The time course of preconditioning protection consists of an initial early phase (1–2 h) and a subsequent later phase (12–24 h) lasting up to 3–4 days that requires synthesis of new proteins following genetic reprogramming of myocytes [42-44]. It may be that protective effects of DDFPe, which extend into the subacute phase of ischemia,

are mediated via complex second messenger preconditioning pathways that involve such components as adenosine, adenosine receptors, the epsilon isoform of protein kinase C, mitoK_{ATP}, and the paradoxical protective role of ROS [44-48]. If systemic administration of DDFPe can increase ROS production, further studies are needed to elucidate whether a single administration of DDFPe may be more optimal in stimulating a protective preconditioning effect, as compared to repeated dosing, where we would focus on the later phase myocardial protective effect as the measured outcome. Carried further, our findings, presented here, may lead to novel approaches for treating myocardial ischemia.

The molecular imaging method to assess DDFPe cardioprotection was based on detection of externalized PE on apoptotic cell surfaces as a cell stress response [49]. Like PS, PE is a constituent of the inner leaflet of the plasma membrane, with little presence on the surface of typical viable cells [50]. When PE becomes accessible to the extracellular milieu due to compromised plasma membrane integrity, ^{99m}Tc-duramycin serves as a molecular probe of PE and is capable of localizing myocytic cell death, including apoptosis and early necrosis [22,23]. Upon binding to PE, duramycin becomes deposited in cellular membranes [21,51]. In contrast to externalized PS, which serves as a signaling mechanism for scavenging of dying cells, the externalization of PE is likely to contribute to reorganization of membrane structure and cytoskeletal components. Given its low molecular weight, high stability, high binding affinity, and specificity, ^{99m}Tc-duramycin may have a great potential for noninvasively measuring myocardial cell death and to assess timely therapeutic intervention aimed at preventing progressive myocardial infarction. This study serves as evidence for this potential utilization of ^{99m}Tc-duramycin, at least for research purposes.

^{99m}Tc-duramycin has been tested in a variety of animal models to investigate its cell death targeting properties, such as myocardial and cerebral ischemia-reperfusion injury, and cancer cell therapeutic responses [22,24,52]. In the present study, we further collected dynamic SPECT cardiac imaging data in the animals of Group I to delineate the feasibility of ^{99m}Tc-duramycin imaging to assess ongoing myocytic injury. Unlike a slow washout pattern of ^{99m}Tc-glucurate, which is an infarct-avid imaging agent based on cell necrosis [26], the accumulation of ^{99m}Tc-duramycin in reperfused myocardium was dynamically increased over the 2 h following tracer administration. This progressive uptake profile of ^{99m}Tc-duramycin is probably due to continuous apoptotic cell death promoted by reperfusion injury. Thus, without euthanizing animals, myocardial injury can be quantified and monitored dynamically using ^{99m}Tc-duramycin. To our knowledge, the in vivo kinetics of ^{99m}Tc-duramycin has not been investigated previously with dynamic high-resolution SPECT imaging in living animals. We did not perform dynamic imaging in Group II-V in the current study because we were concerned that continuous use of Isoflurane anesthesia mixed with oxygen over the 2-h image acquisition period might alter the cardioprotection effects of DDFPe and confound our experimental design. We therefore collected in vivo and ex vivo data from an extra group of rats treated with DDFPe at early myocardial ischemia and at 24-h post reperfusion to account for the lack of dynamic observations of ^{99m}Tc-duramycin uptake in the myocardium. After 24-h reperfusion, ^{99m}Tc-duramycin radioactivity in the necrotic myocardium, defined by a lack of TTC staining, did not show a significant difference between the DDFPe-treated and saline-controlled groups. The radioactivity in the entire IAR, including ischemic viable and ischemic nonviable myocardium, showed a

significant greater increase in the saline control group than that in the DDFPe-treated group. This biodistribution pattern of ^{99m}Tc -duramycin indicates that ischemic viable myocytic cells in the DDFPe-protected myocardium may become less apoptotic or less necrotic following 24 h of reperfusion.

5. Conclusions

This is the first study to assess the protective potential of DDFPe administered after a period of ischemia in an acute ischemia-reperfusion animal MI model. A protective effect against myocardial ischemic-reperfusion injury was achieved by a single dose of DDFPe mediated during a 45-min ischemic episode, followed by 2-h and 24-h reperfusion. The significant tolerance to myocardial ischemia-reperfusion injury could be consistently assessed by ^{99m}Tc -duramycin SPECT imaging and postmortem histopathological analysis. The single dose of DDFPe protected ischemic-reperfused hearts beyond oxygen transport potential of this agent suggests the possibility of other mechanisms associated with the cardioprotection. Further investigation of DDFPe as a cardioprotective agent at repeated doses and later time administration after myocardial ischemia is warranted. Other important components of future work need to be focused on the mechanism of DDFPe in attenuating ischemia-reperfusion injury as observed at the late phase of this study, as well as the long-term impact of DDFPe therapy on post-MI repair.

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Fig. 1.

Representative FastSPECT II dynamic images (serial coronal tomographic slices) of ^{99m}Tc -duramycin in a rat heart with 45-min ischemia and 2-h reperfusion. The rat was anesthetized with 1.0% isoflurane and received intravenous injection of 66.6 MBq ^{99m}Tc -duramycin. The number listed at the lower right corner of each image refers to the time after tracer injection. Image data were acquired every min for 10 min after tracer injection, and then 5-min every 15 min up to 2 h post-injection. Radioactivity distributed predominantly in the cardiac blood pool (white arrows) at 1 min. Based on the early image of cardiac blood pool, remote LV wall (septal wall) with no coronary occlusion was defined as marked by light blue arrow heads on 1-min image. Focal radioactive uptake (hot-spot) marked by green arrows on 120-min image was gradually visualized in the LV anterolateral wall starting at 15 min and remained well-visualizable at 2 h.

Fig. 2.

A and B: FastSPECT II images at 1-min (A) and 120-min (B) post-injection of ^{99m}Tc -duramycin picked from Fig. 1. The cardiac blood pool and hot-spot uptake of ^{99m}Tc -duramycin are marked by white arrows and green arrows, respectively. **C:** Co-registration of 1-min and 120-min images, on which the 120-min image is transformed to greenscale to show the association of hot-spot to the heart. **D, E, and F:** ROI establishments in light blue on the 1-min and 120-min images for quantitative analyses of the cardiac blood pool (D), remote LV septal wall (E), and the hot-spot of ischemic anterolateral wall (F), as well as the liver.

Fig. 3.
Top: ^{99m}Tc -duramycin TACs in normal myocardium, ischemic area (hot-spot), cardiac blood pool, and the liver in the rats of Group I. **Bottom:** Averaged radioactive ratio of ischemic area (hot-spots) to remote healthy myocardium. * Significant difference between the LCA hot-spot of the ischemic area and the normal zones from 5 min to 2 h ($P < 0.05$).

Fig. 4. Results of nonlinear Regression - Dynamic Fitting of the TACs generated from the cardiac blood pool, left ventricular normal and ischemic areas on FastSPECT II images of I/R rat hearts.

Fig. 5. ^{99m}Tc -duramycin FastSPECT II cardiac images (transverse slices, 0.5 mm/slice) of two representative rats with 45-min LCA ligation and 24-h reperfusion. **Top panel:** The rat was treated with DDFPe. **Bottom panel:** the rat received saline as control. The rats were anesthetized with 1.0% isoflurane and received intravenous injection of 77.7 MBq and 70.3 MBq ^{99m}Tc -duramycin, respectively. Image data were acquired for 10 min at 2 h after tracer injection. Hot-spot radioactive uptake was visualized in the anterolateral wall of the left ventricle in both rats. The rat protected by DDFPe showed lower radioactive uptake in the whole heart compared to the heart unprotected.

Fig. 6.

Top row: The delineation of whole LV wall on coronal, sagittal, and transverse slices of ^{99m}Tc -duramycin FastSPECT II reconstruction in the rat received saline as control in Fig. 5.

Bottom row: Corresponding ROI establishments over the hot-spots exhibited on the coronal, sagittal, and transverse slices for quantitative hot-spot measurement.

Fig. 7. Results of quantitative analysis of ^{99m}Tc -duramycin FastSPECT II images in the I/R rat hearts received DDFPe and saline treatment. **Top:** Volume of radioactive uptake (hot-spot) in the LV wall. **Bottom:** Ratio of hot-spot to remote healthy zone radioactivity. * $P < 0.05$ compared to saline control group.

Fig. 8.

^{99m}Tc-duramycin biodistribution at 2-h after radiotracer administration in dissected left ventricles. **R2h:** ^{99m}Tc-duramycin was injected at 2-h post-reperfusion in Group II and IV treated by DDFPe; **R24h:** ^{99m}Tc-duramycin was injected at 24-h after reperfusion in Group III and V received carrier vehicle (saline). * $P < 0.05$ compared to the saline control group at 2-h reperfusion; † $P < 0.05$ relative to the control group at 24-h reperfusion.

Fig. 9. ^{99m}Tc -duramycin autoradiograph images (A), Evans blue (B) and TTC staining (C) of LV slices of a representative DDFPe-treated ischemic rat heart with 2-h reperfusion when ^{99m}Tc -duramycin was injected. **D:** Results (mean $\pm$ SEM.) of postmortem measurements of LV IAR (%LV), infarct (%LV), and infarct size normalized by IAR size (%IAR) in Group II (DDFPe/R2h), Group III (Saline/R2h), Group IV (DDFPe/R24h), and Group V (Saline/R24h). * $P < 0.05$ compared to Saline/R2h. † $P < 0.05$ relative to Saline/R24h.

Fig. 10.

Histopathology of LV sections (5 μ M) with H&E staining in a representative ischemic rat heart with 2-h reperfusion received saline as vehicle control. **A:** The tissue section (40 $\times$) reveals myocardial infarct in the anterolateral wall of LV with hypereosinophilic characteristics. **B:** Remote healthy area (200 $\times$). **C:** Infarct zone of the LV section (200 $\times$) shows myocytic injury and inflammatory cell infiltration.

Fig. 11. Photomicrographs (400 $\times$) of LV sections (5 μ M) stained with active caspase-3 antibody from two ischemic rat hearts with 2-h reperfusion. **A:** Representative section from a rat treated with DDFPe. **B:** Section from a rat received saline as carrier vehicle. Cleaved caspase-3 positive cells were observed in the ischemic area-at-risk (IAR), in which normal cell nuclei were stained pale blue, while the apoptotic cardiomyocyte nuclei were stained brown. The LV section with DDFPe treatment showed fewer cells stained positively for caspase-3 compared with the control section.

Table 1**Biodistribution of ^{99m}Tc -duramycin (%ID/g) (mean $\pm$ s.e.m).**

Tissue	Group II DDFPe/R2h	Group III DDFPe/R24h	Group IV Saline/R2h	Group V Saline/R24h
Blood	0.13 $\pm$ 0.02	0.12 $\pm$ 0.02	0.20 $\pm$ 0.02	0.12 $\pm$ 0.03
Heart	0.83 $\pm$ 0.14 [*]	0.76 $\pm$ 0.09	1.34 $\pm$ 0.11 [‡]	0.73 $\pm$ 0.05
Lung	0.24 $\pm$ 0.06	0.18 $\pm$ 0.03	0.25 $\pm$ 0.04	0.20 $\pm$ 0.06
Liver	1.09 $\pm$ 0.43	1.94 $\pm$ 0.26	1.21 $\pm$ 0.26	2.10 $\pm$ 0.22
Spleen	0.49 $\pm$ 0.11	0.79 $\pm$ 0.10	0.54 $\pm$ 0.10	1.10 $\pm$ 0.15
Stomach	0.10 $\pm$ 0.02	0.06 $\pm$ 0.03	0.08 $\pm$ 0.02	0.09 $\pm$ 0.03
Small Intestine	0.32 $\pm$ 0.04	0.33 $\pm$ 0.06	0.39 $\pm$ 0.05	0.33 $\pm$ 0.07
Large Intestine	0.05 $\pm$ 0.01	0.23 $\pm$ 0.05	0.09 $\pm$ 0.04	0.23 $\pm$ 0.05
Kidneys	5.64 $\pm$ 0.36	6.87 $\pm$ 0.47	8.17 $\pm$ 1.80	5.94 $\pm$ 0.49
Skin	0.12 $\pm$ 0.02	0.16 $\pm$ 0.02	0.39 $\pm$ 0.27	0.13 $\pm$ 0.02
Muscle	0.01 $\pm$ 0.00	0.07 $\pm$ 0.05	0.02 $\pm$ 0.00	0.02 $\pm$ 0.00

R2h: reperfusion for 2 h; R24h: reperfusion for 24 h.

^{*} $P < 0.01$ compared to Group IV.[‡] $P < 0.01$ compared to Group V.

Table 2Radioactive ratios of ischemic to normal myocardium (mean $\pm$ s.e.m).

Tissue	Group II DDFPe/R2h	Group III DDFPe/R24h	Group IV Saline/R2h	Group V Saline/R24h
Infarct/Normal	15.74 $\pm$ 5.35 *	8.53 $\pm$ 1.48 ‡	27.27 $\pm$ 4.62	14.09 $\pm$ 1.78
IAR/Normal	11.09 $\pm$ 2.79 *	6.61 $\pm$ 0.81 ‡	21.28 $\pm$ 3.29	13.52 $\pm$ 2.05

* $P < 0.01$ compared to Group IV.‡ $P < 0.01$ compared to Group V.