Approach to Assessing Myocardial Perfusion in Rats Using Static [13N]-Ammonia Images and a Small-Animal PET

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Abstract

Purpose: Semi-quantitative, static positron emission tomography (PET) has been used to perform an initial approach to the assessment of [13N]-ammonia perfusion studies aimed to elucidating the effect of injecting human embryonic stem cell-derived (hES) hemangioblasts on infarcted rat hearts.

Procedures: Female NIH nude rats underwent occlusion of the left anterior descending coronary artery for 30 min before reperfusion. Either one million hES-derived hemangioblasts ($n=5$) or control media ($n=4$) were injected into the site of the infarct 1 day post-myocardial infarction (MI) under high-resolution echocardiography guidance. PET imaging was performed 6 weeks after MI induction, and uptake polar maps were created by sampling the left ventricle at equidistant slices from the base to the apex and measuring the average myocardium value at three contiguous voxels to minimize partial volume effects. Statistical comparison between treatment and control groups was done with a Mann–Whitney U test.

Results: Myocardium uptake ratios for treated and untreated subjects show statistically significant difference (98% certainty).

Conclusions: The straightforward procedure described here (similar to those commonly used in clinical routine) was sufficient to yield statistically significant perfusion differences between the treated and untreated animals despite the small sample size.

Key words: Small-animal PET, Myocardial perfusion, hES hemangioblasts, [13N]-Ammonia

Introduction

Positron emission tomography (PET) using [13N]-ammonia has been used to evaluate myocardial blood flow in experiments of heterotopic cardiac transplantation in the rat [1]. Experiments on imaging reporter gene expression for noninvasive monitoring of gene therapy have also made use

of [13N]-ammonia PET images to assess myocardial perfusion [2, 3]. In this experiment, we use semi-quantitative [13N]-ammonia perfusion imaging to assess the effect on myocardial blood flow of injecting human embryonic stem cell-derived (hES) hemangioblasts into the infarct area. We want to assess whether the hemangioblasts could form new blood vessels that would result in improvements in myocardial perfusion following myocardial infarction in a rat model.

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Methods

Hemangioblasts Culture

hESC culture was prepared according to [4]. The hESC lines (Advanced Cell Technology, Alameda, CA, USA) were grown on mitomycin C treated mouse embryonic fibroblasts in complete hESC media until they reached 80% confluence. hESC were dissociated and collected by centrifuge at 1,000 rpm for 5 min. To induce hemangioblasts precursor formation, cells were plated in ultra low dishes with the addition of bone morphogenic protein (BMP) 4 and VEGF165 (50 ng/ml, R&D System) and cultured in 5% CO₂. After 48 h, half the media were changed and fresh media with the same final concentration of BMP 4 and VEGF were added, plus stem cell factor thrombopoietin and Flt3 ligand (20 ng/ml; R&D system). Embryonic bodies were collected and dissociated by EDTA. Single cell suspensions were resuspended in Stemline II medium and plated in blast colony expansion medium. Hemangioblasts were collected 6–7 days after replating.

Either one million hES derived hemangioblasts ($n = 5$) or control media ($n = 4$) in a volume of 50 μ l were injected into the infarct site 1 day post myocardial infarction (MI) under high resolution echocardiography guidance (VisualSonic, Toronto, ON, Canada) according to the methodology described in [5]. This echo guided percutaneous technique ensures that intramyocardial injections of the hES derived hemangioblasts occur in the specific desired region of the myocardial wall, and it also confirms that the material remains in the myocardium.

PET imaging was performed 6 weeks after treatment as described below. All experiments in this study were performed in accordance with the guidelines for animal research from the National Institutes of Health (NIH publication 85-23, revised 1985) and were approved by the Committee on Animal Research at the University of California, San Francisco.

Animal Model of Acute Myocardial Ischemia and Reperfusion

A rat model of reversible myocardial ischemia and reperfusion was used as previously described [6]. Nine female NIH nude rats (200–300 g) underwent occlusion of the left anterior descending coronary artery for 30 min before reperfusion, following a protocol previously described [7, 8]. The animals were anesthetized with 4% isoflurane inhalant with an oxygen output of 0.8–1.0 l/min in an induction chamber, and anesthesia was maintained with 1.5–2.0% isoflurane inhalant in oxygen mixed with room air, ventilated via oral intubation with a PE240 polyethylene tubing using a Harvard rodent respirator (tidal volume 0.5 to 1.5 ml; respiratory rate 95 to 105 breaths/min). A midline sternotomy was performed under sterile procedure, and a reversible coronary artery snare occluder was placed around the proximal left coronary artery (LCA) by using a 7 O TICRON cardiopoint needle with suture loop. After the occlusion, the presence of myocardial ischemia was confirmed by regional cyanosis. LCA occlusion lasted for 25–30 min. Based on our experience [7], this time period of occlusion leads to a sizable myocardial infarction. After this time period, the snare was

Fig. 1. Sprague Dawley rat NH3 myocardium perfusion image (left) compared to a NIH rat (right). Inferior and apical sides of the NIH rat heart lies directly on top of the liver (red area at the bottom of the images) causing that a great amount of activity from the liver spills over the myocardium.

Fig. 2. QA phantom image analysis: ROI were drawn around the hot rods, and their activity was calculated as the mean of the five hottest points in the ROI. The 100% recovery coefficient (0.11 kBq/cc) was measured on a uniform background ROI (9 mm diameter) free of spillover effects.

released, and reperfusion was verified by hyperemia. The rats were then allowed to stabilize for 10 min, and the chest was closed by suturing down layers. Air was evacuated from the thoracic cavity. Throughout the experiments, ECG was continuously monitored and core body temperature was monitored by a rectal thermometer and kept constant by heating pads and lamps at $37 \pm 0.5^\circ\text{C}$. Any episodes of ventricular tachycardia or fibrillation which might occur during LAD occlusion were immediately stopped by mechanical procedures. Animals were placed under heating lamp for their full recovery, and 0.1 ml of 2.27% enrofloxacin (Baytril) was administered I.M. thereafter. The animals were allowed to recover for 1 day before therapeutic injection.

PET Imaging

Each animal underwent a tail vein injection of $[^{13}\text{N}]$ ammonia (mean 3.8 ± 0.4 mCi, less than 1.5 ml) 42 days following the induction of the MI. Rats were prepared for imaging under isoflurane anesthesia (Abbott Laboratories) delivered through a nose cone at a concentration of 1.5–2.0% volume and 1.0 l/min oxygen flow. A venous cannula was placed in the tail vein for the administration of $[^{13}\text{N}]$ ammonia.

Imaging experiments were performed using a dedicated Inveon (Siemens) small animal PET scanner [9]. During imaging, the animal rested supine on the scanner bed and was kept warm with a heating pad. A 15 min imaging acquisition started 4 to 10 min after the intravenous bolus administration of $[^{13}\text{N}]$ ammonia.

A static image was reconstructed (128×128 matrix with a 0.4×0.4 mm² pixel size, 0.75 mm slice thickness) using a statistical maximum a posteriori probability algorithm (3D MAP) with reconstruction parameters optimized for uniform resolution. This algorithm implements a local sinogram blurring operator that models and corrects for detector response effects, photon pair non colinearity, crystal penetration, inter crystal scatter, and the effects of the block detector geometry [10]. The resulting effective spatial resolution was measured with a phantom, as described below.

Data Analysis

Imaging data were analyzed with the PMOD program (PMOD Technologies Ltd., Zurich, Switzerland). The left ventricle

myocardium was reoriented into short and long axis slices and automatically segmented in PMOD, manually readjusting the segmentation when necessary. An uptake polar map was created by sampling the left ventricle (LV) at 20 equidistant slices from the base to the apex, with 36 samples per slice around the LV perimeter for all slices except the apex, for which a single sample was used. The value at each segment of the polar map was obtained by measuring the average myocardium value at three contiguous voxels (the standard procedure in PMOD to minimize partial volume effects) along the short radial axis at its intersection with the LV segmentation. The perfusion analysis was carried out on this polar map.

Myocardial uptake ratios (infarcted tissue uptake divided by normal tissue uptake) were measured using a supervised (automatic initial guess followed by a manual correction when needed) segmentation on the polar map; infarcted tissue was defined as the area which includes the voxel of minimum uptake, while uptake for normal tissue was measured at the contra lateral (diametrically opposed) region of the infarct area, taking care to avoid regions affected by liver spillover. Defect size was quantified as the percentage of myocardium area below 75% of the maximum uptake on the whole polar map. This maximum uptake value was calculated by averaging the five highest values of the polar map

Fig. 3. Polar map of a representative $[^{13}\text{N}]$ -ammonia perfusion study on a NIH rat. A The area affected by the liver spill over, B the area selected as “normal”, and C is the area used to estimate the infarct uptake.

Fig. 4. Perfusion and infarct size for treated and untreated subjects ($n=9$, five treated, four control). The two-tailed Mann–Whitney U test result shows statistically significant difference for the uptake ratios (98% certainty), but not for the infarct size.

not affected by liver spillover. Finally, Masson’s trichrome staining in the excised hearts was used to qualitatively determine infarct size.

Calibration

Since this experiment was carried out under non ideal imaging conditions (no cardiac or respiratory gating, no dynamic acquisition, no basal data acquisition), in order to properly model the spillover effects, a NEMA NU 4 QA phantom [11] was used to obtain a realistic estimation of the effective resolution and recovery coefficients on the resulting images. For that purpose, the phantom was loaded with a solution of $[^{13}\text{N}]$ ammonia at a concentration that yielded an acquisition count rate similar to that obtained in the animal experiments. Phantom and animal images were reconstructed using the same algorithm and parameter settings. Images of axial slices through the hot rods section and the large uniform section of the phantom were analyzed to derive spatial resolution and recovery coefficients. The activity of each of the five hot rods (from 1 to 5 mm diameter) was calculated as the mean activity of the hottest five voxels inside the ROI (ROI radius = rod radius plus 2 mm). The same measurement was taken from a 9 mm diameter ROI placed in the middle of the uniform area of the phantom filled with the same activity and that result was used as the basis to define 100% recovery coefficient. For our purposes, rather than measuring the system resolution following a protocol like the one described in

[11], we estimated an “effective resolution” by comparing the measured size of the hot rod with its true size for a rod with a recovery coefficient of > 0.7 .

Using the previous results, a simple simulation of the heart and liver activity distribution and the spillover between them was done in order to assess the validity of the proposed analysis; LV was simulated as a 2 mm thick annulus, 9 mm diameter, and with an arbitrary activity for normal (healthy) tissue defined as 100%. A defect (50% activity) was induced on a 90° sector, blood background inside the LV was set to 20% while the outside background was set to 20%, and the liver activity was 125%. The effect of the system resolution was simulated as a Gaussian blurring defined by the experimentally measured effective resolution as described above. This effective resolution is the result of the convolution of the system spatial resolution, scatter, and isotope positron range. In order to assess the effect of the contrast recovery of the image reconstruction algorithm, different profiles across this phantom were drawn including or not the liver and its vicinity.

Statistical Methods

Uptake ratio and defect size data were expressed as mean $\pm$ standard deviation. The comparison between treatment and control groups was carried out using a two tailed Mann Whitney U test.

Results

The anatomy of the animals studied introduced an additional difficulty to the PET imaging, since in this breed the heart lies directly on top of the liver. Because more activity accumulated in the liver than in the myocardium after 15 min of image acquisition, the spillover of the first over the second becomes predominant on the apical and inferior-apical regions of the heart, rendering those areas unusable for quantitative or semi-quantitative analysis purposes (Fig. 1). Recovery coefficient analysis showed (Fig. 2) that for our experimental setup, the combined effect of system resolution (including all the undesirable effects described in the “PET Imaging” section) and cardiac movement results in a >2 -mm effective resolution.

The simulations showed that with a careful selection of the ROIs, it is possible to compute semi-quantitative uptake

Fig. 5. The Masson’s trichrome staining images for a control (left) and a treated animal (right).

ratios with negligible effect of the spillover contamination if profiles are drawn far from the liver area of influence. The 3D-MAP reconstruction algorithm introduces a resolution recovery that results in images in which the areas far from the liver were not contaminated by its high activity. Thus, infarcted tissue uptake measurements on the anterior side of the myocardium, far away from the liver, were reliable. The same condition applies to the lateral and anterior-septum regions (Fig. 3).

Fig. 4 shows a bar graph of the myocardium uptake ratios and infarct size for treated and untreated subjects ($n=9$, 5 treated, four control). In order to find out if it is possible to differentiate the controls from the treated animals, a two-tailed Mann–Whitney U test was applied. The results show statistically significant difference for the uptake ratios (98% certainty), but not for the infarct size.

Finally, Fig. 5 shows the Masson's trichrome staining images for a control and a treated animal. These preparations were used to qualitatively confirm the location and extension of the infarcts.

Discussion and Conclusion

An approach to semi-quantitatively assess myocardial perfusion has been tested using $[^{13}\text{N}]$ -ammonia in nine animals with induced MI. Despite the difficulties for imaging small animals with this pathology, we have been able to define a procedure that seems to provide reliable results which are in agreement with the visual qualitative assessment. According to these results, we were able to separate the two groups of animals included in the study (treated and non-treated) reaching statistical significance in the case of the uptake ratios, which can be assumed to reflect defect severity. Although the infarct size was apparently smaller in the treated group, this difference did not reach statistical significance. Two treated animals showed an infarct size larger than those observed in most of the control animals. Because the baseline infarct size before the therapy was applied was unknown, it was not possible to determine whether the treatment in those animals was unsuccessful or if their initial infarct size was too large to show an incremental treatment effect. This reinforces the idea that for a better assessment of the effect of the treatment, a baseline perfusion measurement would be necessary, particularly if the sample size is small, as it is our case. Including basal data would have provided a more statistically powerful longitudinal comparison for the post treatment perfusion measurement. Although quantitative blood flow measurement derived from kinetic

modeling usually provides more robust results, experimental restrictions prevented the use of dynamic acquisitions. Nonetheless and even considering that the sample size used in this experiment was small, the simple procedure described here (similar to those commonly used in clinical routine) was sufficient to yield statistically significant perfusion differences between the treated and untreated animals. Therefore, we can conclude that the proposed technique to assess myocardial perfusion in rats using $[^{13}\text{N}]$ -ammonia images and a small animal PET would provide reliable results in experiments involving larger cohorts and preferably including a basal study before treatment.

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Conflict of Interest. The authors declare that they have no conflict of interest.

References

- Hoff SJ, Stewart JR, Frist WH et al (1992) Noninvasive detection of heart transplant rejection with positron emission scintigraphy. *Ann Thorac Surg* 53(4):572–577
- Wu JC, Inubushi M, Sundaresan G, Schelbert HR, Gambhir SS (2002) Positron emission tomography imaging of cardiac reporter gene expression in living rats. *Circulation* 106(2):180–183
- Wu JC, Chen IY, Sundaresan G et al (2003) Molecular imaging of cardiac cell transplantation in living animals using optical bioluminescence and positron emission tomography. *Circulation* 108(11):1302–1305
- Lu S, Ivanova Y, Feng Q, Luo C, Lanza R (2009) Hemangioblasts from human embryonic stem cells generate multilayered blood vessels with functional smooth muscle cells. *Regen Med* 4(1):37–47
- Springer ML, Sievers RE, Viswanathan MN et al (2005) Closed-chest cell injections into mouse myocardium guided by high-resolution echocardiography. *Am J Physiol Heart Circ Physiol* 289(3):H1307–H1314
- Piot CA, Padmanaban D, Ursell PC, Sievers RE, Wolfe CL (1997) Ischemic preconditioning decreases apoptosis in rat hearts *in vivo*. *Circulation* 96:1598–1604
- Huang NF, Sievers PJS et al (2006) A rodent model of myocardial infarction for testing the efficacy of cells and polymers for myocardial reconstruction. *Nat Protocols* 1(3):1596–1609
- Yu J, Christman K, Chin E et al (2009) Restoration of left ventricular geometry and improvement of left ventricular function in a rodent model of chronic ischemic cardiomyopathy. *J Thor Cardiovasc Surg* 137(1):180–187
- Visser EP, Disselhorst JA, Brom M et al (2009) Spatial resolution and sensitivity of the Inveon small-animal PET scanner. *J Nucl Med* 50(1):139–147
- Cho S, Li Q, Ahn S, Bai B, Leahy RM (2007) Iterative image reconstruction using inverse Fourier rebinning for fully 3-D PET. *IEEE Trans Med Imaging* 26(5):745–756
- National Electrical Manufacturers Association (2008) NEMA 2008 performance measurements for small animal positron emission tomographs. NEMA Standards. Volume Publication NU 4-2008. Rosslyn, VA.