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Comparison of Neutron Activated and Radiolabeled Microsphere Methods for Measurement of Transmural Myocardial Blood Flow in Dogs

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***Abstract. Background*: The 'gold standard' radioactive microsphere (RM) technique for measurement of organ blood flow under various experimental conditions is inaccessible to many researchers due to increasing environmental concerns regarding safety and disposal of low-level radioactive waste materials. A new method using neutron activated microspheres (NAM) has recently been described.**

***Methods*: We compared regional myocardial blood flows using the new formulation STERIspheres™ (NAM; 15.0 ± 0.1 [SD] μm; density 1.5 gr/mL) with RM (15.0 ± 0.1 [SD] μm; density 1.5 gr/mL) under different experimental conditions during acute ischemia-reperfusion injury in dogs. Random paired combinations of four different RM and NAM were co-injected into the left atrium during autoregulation, coronary occlusion and flow-mediated hyperemia (reperfusion) in the same animal. The left ventricle was divided into non-ischemic and ischemic regions and further subdivided into endocardial, mid-myocardial and epicardial portions. After gamma-counting, blood and myocardial tissue samples ($n = 180$) were dried and then shipped to a core facility for neutron activation and analysis. NAM-RM blood flow data were directly compared by ANOVA and regression analysis; Bland and Altman analysis was also performed to assess mean differences in blood flow with NAM-RM.**

***Results*: A direct relation for blood flow between NAM-RM was observed; the slope of the relation (1.17 RM ± 0.04 [SEE]) was different from unity but the intercept (0.06 ± 0.06 [SEE]) was not different from the origin. Intermethod mean differences were minimal between NAM-RM in the low to normal range of blood flow and were increased at the higher blood flow levels the latter being of minor physiological consequence. A direct relation for endo/epicardial blood flow ratios between NAM-RM was also observed; the slope of the relation (0.98 RM ± 0.04 [SEE]) and the intercept (0.03 ± 0.06 [SEE]) were not different from unity or the origin, respectively.**

***Conclusions*: Results show that in addition to limiting production of radioactive waste materials, NAM accurately measure myocardial blood flow, endocardial/epicardial and ischemic/non-ischemic blood flow distributions over a wide range.**

***Abbreviated abstract.* We compared myocardial blood flows using paired combinations of neutron activated (NAM) and the 'gold standard' radiolabeled microspheres (RM) co-injected during autoregulation, coronary occlusion and flow-mediated hyperemia in an *in situ* canine ischemia-reperfusion preparation. A direct relation for blood flow and endo/epicardial blood flow ratios between NAM-RM was observed; intermethod mean differences between NAM-RM were minimal in the low to normal blood flow range but increased at higher blood flow levels. These results indicate that NAM accurately measure myocardial blood flow and its transmural distribution in addition to limiting unnecessary production of radioactive laboratory waste products.**

***Key Words.* neutron activated microspheres, radioactive microspheres, blood flow, ischemia-reperfusion**

Introduction

Accurate evaluation of blood flow across the left ventricular wall is not yet possible in patients, however, distribution of myocardial blood flow can be evaluated in *in situ* animal models using microspheres, the gold standard being radioactive microspheres (RM) [1,2]. Increasing environmental concerns with regard to low-level radioactive waste materials, cost and reduced availability of isotopes has stimulated considerable efforts to search for a suitable replacement [3,4] in particular for evaluation of blood flow in the microcirculation. Several alternatives to RM have been described [4–8], the most recent being stable labelled microspheres that are non-radioactive but contain isotopic tracers within each sphere that can be activated by neutron irradiation [9]. These microspheres are used in a manner identical to the RM and share similar physical attributes [6,10,11]. A

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study using first-generation neutron activated microspheres (NAM) to measure myocardial perfusion in an *in situ* rabbit preparation of myocardial stunning has been reported [9]. In the present study we evaluated myocardial blood flow and its distribution in a large animal model of ischemia-reperfusion injury using the second generation NAM (STERIspheres^{TM}) co-injected with RM.

Materials and Methods

Experiments were conducted in accordance with the "*Guide to the Care and Use of Experimental Animals*" (vols. 1 and 2) of the Canadian Council on Animal Care; the Laval University Animal Ethics Committee approved these studies.

Surgical preparation

Adult mongrel dogs of either sex (weight 20–25 Kg) were pre-medicated with acepromazine maleate (ACEVET; 0.5 mg/Kg IM, Vétoquinol Inc. Lavaltrie, CAN) and then anesthetized using sodium pentobarbital (25 mg/Kg IV). Dogs were intubated and mechanically ventilated; end-expiratory pressure was maintained at 5–7 cm H_2O. Arterial blood gases were monitored throughout the study and respirator settings were adjusted to maintain PO_2 and PCO_2 values within the physiological range. During the experiments body temperature was monitored by a rectal thermometer and kept between 37.5–38.5°C by a water-jacketed Micro-Temp heating unit (Zimmer, Dover, OH, U.S.A.).

The heart was exposed through a left thoracotomy and suspended in a pericardial cradle. Polyethylene catheters were introduced into the internal thoracic artery (for withdrawal of reference arterial blood samples), the left atrium (for injection of microspheres) and the right femoral artery (to measure aortic pressure) and vein (for administration of drugs and fluids). A 5F micro-tipped pressure transducer (MPC500, Inter V Medical Inc., Montréal, CAN) was placed in the LV cavity through the apex to measure LV pressure. A segment of the left circumflex coronary artery was dissected free distal to the first marginal branch and a flowprobe (3SB; Transonic Systems Inc., Ithaca, NY, U.S.A.) was positioned; a snare occluder was placed immediately distal to the flowprobe.

Standard lead II of the scalar EKG was used to assess heart rate; LV pressure was recorded continuously during the study. Phasic arterial pressures were measured with Gould P23XL pressure transducers (calibrated to the fluid-filled Pigtail catheter in the aorta) positioned at mid-chest level. Analog data were recorded on a 12-channel direct writing oscillograph (Yokogawa OR1200A; Electrometers, Dorval, CAN) and on VHS tape using a TEAC XR-510 data recorder.

Experimental protocol

After a 30-min stabilization period myocardial perfusion in the posterior (ischemic zone) and anterior (non-ischemic zone) ventricular wall was assessed by RM and NAM under four experimental conditions: (1) during coronary autoregulation (i.e., with intact coronary tone), (2) 30-min after onset of coronary occlusion, (3) 30-min after onset of reperfusion of the infarct-related artery, and (4) 180-min after onset of reperfusion. All dogs were subjected to the same ischemia-reperfusion protocol consisting of 90-min regional coronary artery occlusion followed by 180-min reperfusion.

Preparation of RM and NAM for regional blood flow determinations

NAM (Sm, La, Yb, Lu; STERIspheres^{TM}, BioPAL Inc., Worcester, MA) were compared with RM (^{141}Ce, ^{85}Sr, ^{95}Nb, ^{46}Sc; NEN-TRAC^{TM}, Perkin Elmer Life Sciences Inc., Boston, MA). RM were made from polystyrene resin, had mean diameters of 15.5 ± 0.1 μm and a specific gravity of 1.4 gm/mL; specific activities of each nuclide were determined. In NAM the isotope label is cross-linked within the polystyrene microsphere matrix; these microspheres had a mean diameter of 15.0 ± 0.1 μm and a specific gravity of 1.5–1.7 gm/mL. To prevent aggregation, microspheres were suspended in saline plus Tween 0.01%; suspension solution for NAM also contained the bacteriostatic agent Thimerosal (0.01%). To ensure a 95% probability that blood flow measurements are within 10% of the true value a minimum of 400 microspheres must be present in each sample [12]. Determination of individual microsphere injectates for these studies was based on the criteria that the heart receives ~5% of total cardiac output; injection of $\sim 2.5 \times 10^6$ RM ensured that the number of spheres in both the reference arterial blood and myocardial tissue samples was sufficient for accurate evaluation of blood flow. Tissues and blood reference samples contained 600 to 2000 microspheres, respectively during baseline and coronary reperfusion experiments. For NAM, $\sim 5 \times 10^6$ microspheres were injected for each blood flow measurement; determination of microsphere injectate was based on the equation (recommended by the manufacturer):

$$Y = 1.2 \times 10^6 + 1.9 \times 10^5 \mathrm{X}$$

where Y is the minimum number of microspheres required and X is the mass of the experimental subject in Kg.

Dual blood flows were determined using RM-NAM co-injected into the left atrium under steady-state hemodynamic conditions. Combinations of the dual tracers were chosen at random; RM plus NAM were placed in an injection vial, thoroughly mixed with a vortex agitator prior to co-injection through the

left atrial cannula and flushed with 15 mL warmed saline. Reference arterial blood was withdrawn with a pump (Masterflex, Cole Palmer, Montreal, CAN) from the internal thoracic artery cannula at a rate of 4.0 mL/min beginning 10-sec before microsphere injection and continuing for 2-min. Myocardial blood flow (expressed in mL/min/g) was calculated using the equation: $Q = (T_{CPM} \times Q_{ref})/(REF_{CPM} \times g)$, where Q is blood flow, T_{CPM} is the number of radioactive counts in the tissue sample, Q_{ref} is the reference flow rate (mL/min), REF_{CPM} is the number of radioactive counts in the reference blood sample and g is the weight of the tissue sample in grams.

Tissue preparation

At the end of each experiment, the left main circumflex artery was re-ligated; 10 mL of Monastral blue dye was injected into the left atrium to delineate the anatomic risk zone. Immediatedly afterwards, under deep anesthesia cardiac arrest was induced by intra-atrial injection of saturated potassium chloride. Both coronary vessels (left anterior descending and left main circumflex arteries) were perfused with sansSa*Li*ne™ at a constant pressure of 80 mmHg for 10-min; sansSa*Li*ne™ is an isotonic, lithium-based reagent designed as a saline substitute for preparing samples for neutron activation analysis (cf. manufacturer's recommendations).

The heart was excised, the left ventricle was fixed in 10% buffered formaldehyde (pH 7.4); after 7 days the left ventricle was cut into 6-mm slices from apex to base parallel to the atrioventricular groove; three transverse sections (beginning with the third most apical slice) were used for the blood flow analysis and were divided into posterior (ischemic zone) and anterior (non-ischemic zone) segments. Each segment was further subdivided into endocardial, mid-myocardial and epicardial portions (cf. Fig. 1) as previously described [13]. Tissue segments (18 from each heart) were placed in preweighed sample vials and then re-weighed; average biopsy weight was 1.06 ± 0.27 gr (mean ± SD; range of 0.48–1.64 gr). RM activity was measured in a multi-channel NaI(TI) gamma-well counter (AutoGamma 5003 Cobra II, Packard Instruments, Meridan, USA) with standard window settings. Samples were counted for 2-min and nuclide activity was corrected for background and decay (overlap corrections were made using the inversion matrix technique). Blood flow (in mL/min/g) was calculated as described previously [14] using PCGERDA computer software (ver. 1.02, Packard Instruments, Meridan, USA). The blood and tissue samples were subsequently stored at 4°C for approximately 4 months to allow for decay of the low-level radioactivity (radioactivity was not detectable in either blood or tissue samples after this time period and were then processed for neutron irradiation).

For the neutron irradiation studies, blood and tissue samples were transferred to trace element-free, polypropylene vials and then dried at 50°C for 48 h; all blood and tissue samples were then shipped to a core laboratory (BioPAL Inc., Worcester, USA) for neutron irradiation and analysis.

Data analysis

Multiple comparisons for cardiac hemodynamic and heart rate data over time were made using ANOVA with repeated measures; the Student-Newman-Keuls multiple range test, with $\alpha \leq 0.05$ was performed on all main-effect means to locate significant differences between interventions. Blood flows at the different experimental time points within ischemic and non-ischemic regions for the co-injected microspheres (NAM-RM) were analyzed using ANOVA with repeated measures. A ratio of ischemic/nonischemic blood flow was determined using mean transmural blood flow within these regions. For

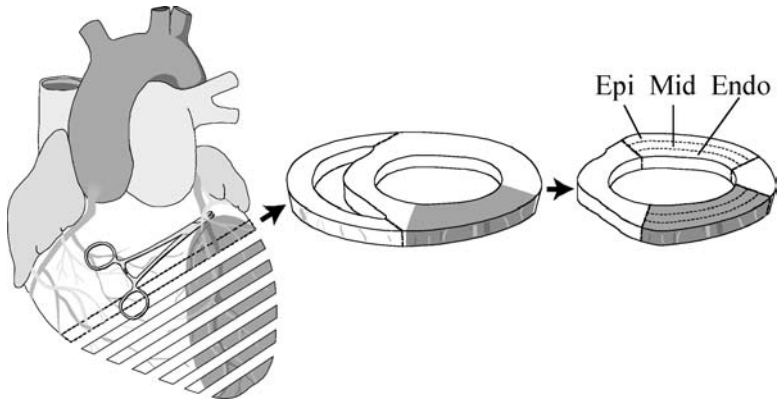

Fig. 1. *Preparation of tissue samples for regional blood flow analysis. The heart was sectioned from apex to base; three sections (beginning with third from apex) were divided into ischemic and non-ischemic zones and then further subdivided into endocardial, mid-myocardial and epicardial pieces.*

intermethod comparisons, a linear regression analysis was applied; regression coefficients (r) and standard error of the estimate (SEE) were determined for the best-fit line. The slope of the regression was compared to unity and the intercept with the origin. To assess differences between the two techniques, the difference (RM minus NAM) between blood flows measured in each piece of tissue was plotted against the mean of the two measurements as described by Bland and Altman [15]. The spatial pattern of blood flow distribution in myocardial biopsies was also quantitated by the coefficient of variation (defined as SD/mean and is related to global flow heterogeneity) [16,17] and is highly dependent on the number of microspheres trapped within the individual tissue sample [17]. Statistical comparisons were done using commercially available computer software (SAS Inc., Cary, USA).

Results

Hemodynamics

Heart rate, ventricular and aortic pressure data during autoregulation (baseline), occlusion and reperfusion are summarized in Table 1. Heart rate was stable during the experimental protocol; LV (systolic) and aortic (systolic/diastolic) pressures decreased significantly during occlusion while diastolic LV pressures increased ($p \leq 0.05$ compared to baseline) during reperfusion. Overall hemodynamic stability during the ischemia-reperfusion protocol is indicated by the relatively minor alterations in the heart rate-arterial pressure product index (indirect indicator of myocardial oxygen demand). The influence of atrial injection of the microspheres on cardiac hemodynamics is illustrated in Figure 2; no change in cardiac hemodynamics or heart rate was observed during microsphere co-injections as the number of microspheres injected intra-atrially was significantly lower than would be required to produce permanent hemodynamic alterations [18,19].

Regional blood flow measurements

Mean blood flow levels in the endocardial, midmyocardial and epicardial tissue samples are summarized in Table 2; no significant differences in blood flow values were observed between

Table 1. *Summary of Cardiac Hemodynamics and Heart Rate*

	HR	LVs	LVd	AoS	AoD	RPI
Baseline	105 ± 18	125 ± 15	8 ± 4	125 ± 15	96 ± 10	13.1 ± 2.3
30-min CO	116 ± 34	111 ± 8*	12 ± 6	111 ± 8*	85 ± 9*	12.8 ± 3.8
REP30	121 ± 26	115 ± 17	14 ± 4*	115 ± 18	91 ± 16	13.8 ± 2.3
REP180	125 ± 26	120 ± 16	13 ± 5*	120 ± 16	93 ± 16	14.9 ± 2.4

Data are means ± 1 SD ($n = 9$ dogs); $^*p \leq 0.05$ vs. Baseline values. CO = coronary occlusion; REP = coronary reperfusion; HR = heart rate (beats per minute); LVs, LVd = left ventricular pressure during systole and diastole (mmHg); AoS, AoD = aortic pressure during systole and diastole (mmHg); RPI = rate-pressure index (beats per minute X mmHg/1000).

Table 2. *Blood Flow in Ischemic and Non-Ischemic Myocardium*

	Ischemic (I)			Nonischemic (NI)			
	Endo	Mid	Epi	Endo	Mid	Epi	I/NI ratio
			Radiolabeled microspheres				
Baseline	0.62 ± 0.09	0.51 ± 0.08	0.41 ± 0.06	0.63 ± 0.15	0.54 ± 0.12	0.45 ± 0.10	0.98 ± 0.09
Occlusion	0.07 ± 0.11*	0.06 ± 0.11*	0.11 ± 0.09*	0.98 ± 0.58	0.78 ± 0.32	0.72 ± 0.47	0.16 ± 0.09
REP30	1.84 ± 1.30	1.05 ± 0.53	0.94 ± 0.72	1.37 ± 0.77	1.18 ± 0.81	0.99 ± 0.90	1.11 ± 0.10
REP180	0.61 ± 0.18	0.46 ± 0.14	0.46 ± 0.13	1.19 ± 0.49	1.01 ± 0.28	0.84 ± 0.36	0.55 ± 0.09
			Neutron activated microspheres				
Baseline	0.66 ± 0.33	0.58 ± 0.25	0.42 ± 0.21	0.70 ± 0.41	0.60 ± 0.33	0.51 ± 0.22	0.94 ± 0.08
Occlusion	0.08 ± 0.08*	0.05 ± 0.07*	0.10 ± 0.08*	1.17 ± 0.74	0.92 ± 0.52	0.83 ± 0.57	0.15 ± 0.08
REP30	2.36 ± 1.92	1.31 ± 0.96	1.26 ± 1.18	1.79 ± 1.30	1.56 ± 1.37	1.68 ± 1.43	1.11 ± 0.11
REP180	0.63 ± 0.39	0.51 ± 0.24	0.58 ± 0.56	1.47 ± 1.18	1.29 ± 1.13	1.01 ± 0.93	0.54 ± 0.08
p(spheres)	NS	NS	NS	NS	NS	NS	NS
p(inter)	0.001	0.001	0.001	0.001	0.001	0.001	0.001

Data are means ± 1 SD ($n = 9$ dogs); baseline values were obtained during autoregulation. Occlusion = 30-min ischemia, REP30 = 30-min reperfusion of the infarct-related artery and REP180 = 180-min reperfusion. Blood flow data are expressed in mL/min/g. I/NI ratio was determined using transmural blood flow data for the respective regions. The Student-Newman-Keuls multiple range test, with $\alpha = 0.05$ was performed on all main-effect means to locate significant differences between interventions and myocardial regional blood flow; $^*p \leq 0.05$ vs. blood flow in corresponding myocardial layers of the non-ischemic region.

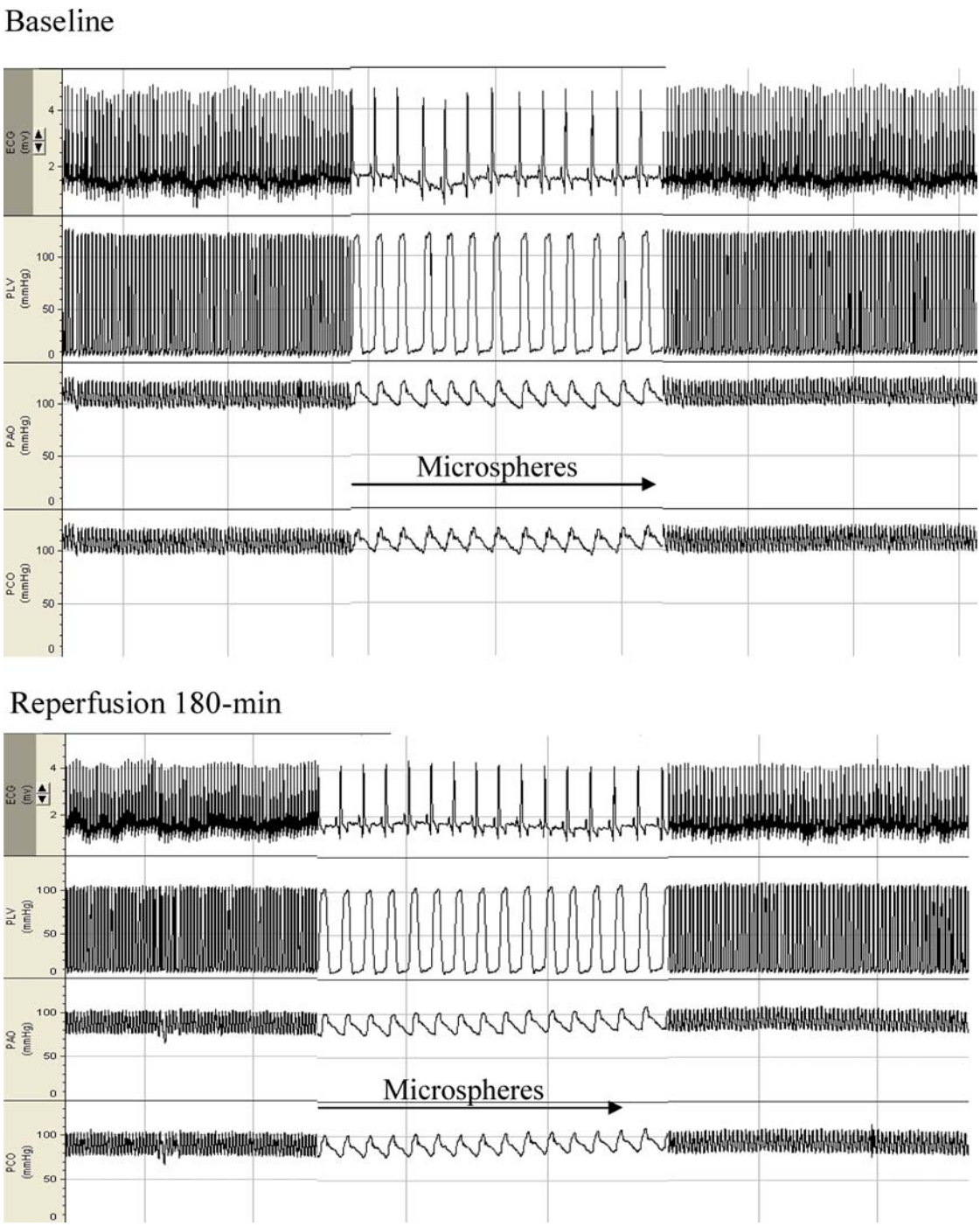

Fig. 2. *Continuous recordings of cardiac hemodynamics and heart rate during intra-atrial co-injections of NAM and RM at baseline and after 180-min coronary reperfusion. EKG indicates cardiac electrical activity, AoP (mmHg), aortic pressure; LVP (mmHg), left ventricular pressure; PCO (mmHg), coronary perfusion pressure and MS, injection of microspheres.*

NAM-RM. Ischemic zone blood flow decreased significantly during coronary occlusion; blood flow in the non-ischemic zone increased during ischemia and remained higher than baseline levels thereafter and is probably due to a compensatory increase in myocardial function and oxygen demand in these tissues [20]. Reperfusion blood flow within the ischemic zone at 30-min was markedly increased but subsequently declined to near baseline values by 180-min; ischemic/nonischemic zone

blood flow ratios were similar for both microsphere types.

The intermethod correlation for NAM-RM blood flow measurements for the pooled data ($n = 180$ samples) is shown in Figure 3A. The slope of the relation was significantly greater than unity ($p = 0.009$) as a result of higher blood flow levels with NAM in the upper range of blood flows; the intercept was not different from zero. Analysis of intermethod differences using the method of Bland and Altman showed no significant offset between NAM and RM (Figure 3B); the mean intermethod error was -0.02 ± 0.43 (mean $\pm$ 2SD). For the endo/epicardial blood flow ratio (Figure 3C), the slope (0.99) and intercept (0.04) of the relation was not statistically different from unity and origin. Analysis of intermethod differences for endo/epicardial blood flow ratios showed no significant offset between NAM and RM (Figure 3D); the mean intermethod error was -0.02 ± 0.32 (mean $\pm$ 2SD).

Discussion

Results of this study demonstrate that regional myocardial blood flow can be accurately measured under different physiological conditions using NAM; at present, up to 14 different measurements of blood flow with STERIspheres^{TM} are possible in the same experimental preparation.

Validation of the NAM method was done by direct comparison of blood flow values to those obtained with simultaneously co-injected RM. Since organ perfusion is highly variable (due to spatial and temporal heterogeneity) it was essential that the different microsphere types be injected simultaneously to evaluate potential differences between flow rates [4,5]. The important finding of the present study is that NAM provides an accurate assessment of regional myocardial blood flow and its transmural distribution; a linear relation between NAM-RM was observed for blood flows over a wide range. The slope of the relation for NAM-RM blood flows differed from unity while the intercept was not different from zero; low to normal flows were comparable between NAM-RM but above the normal range measured flow was higher with NAM (may be related to greater number of NAM in injectate). During early reperfusion (REP30) regional blood flow levels with both NAM and RM were higher than baseline values; although blood flows were greater with NAM these values did not reach a level of statistical significance. Discrepancies in blood flow values particularly at high flow levels between coloured and radiolabeled microspheres in dog hearts has previously been reported[4]. We believe that the observed absolute differences in flow are of minimal physiological significance in this experimental model; however, further studies comparing these two microsphere types under controlled conditions of elevated blood flow (i.e., pharmacologically-induced vasodilatation) are warranted. No differences were observed between NAM-RM for either endo/epicardial or ischemic/non-ischemic tissue blood flow distribution under these experimental conditions.

The RM method is presently considered the standard technique for assessment of organ blood flow under different experimental conditions. Several important limitations of this technique have previously been discussed and have stimulated the search to find a suitable replacement. Major disadvantages of the RM technique include (1) production of radioactive materials that generate increased costs for storage and disposal of biological waste, (2) many precautions are necessary to limit exposure to radioactive materials, and (3) cost of detection equipment including NaI crystal and gamma-counter. On the other hand, NAM offer all the advantages found with RM while limiting potential risk related to utilization of radioactive materials. Major advantages of the NAM technique include (1) generation of reduced amounts of radioactive waste materials, (2) blood flow analysis is undertaken at a central laboratory facility; this improves quality control as neutron activation procedures are consistent for all biological samples and also helps to reduce equipment and manipulation costs and bias between investigators, (3) availability of greater number of neutron activated labels which can help to limit number of animals used and reduce inter-animal variability and (4) reduced time and cost of preparation and analysis of biological samples compared to that required for coloured or fluorescent microspheres.

Potential sources of error related to physical characteristics of microspheres have previously been discussed [1,2,5,21]. Blood flow estimation can be influenced by different physical properties of microspheres including diameter and specific gravity. In the present study there were no differences in diameter between NAM and RM. The close similarity between endo/epicardial (transmural) and ischemic/non-ischemic (regional) blood flow ratios for NAM-RM indicates that the rheological properties of both microsphere types were not different even though there was a slight difference in specific gravity. Similar findings have been reported for coloured microspheres [22].

For this study we used the new formulation STERIspheres^{TM} that contain; (1) higher isotope levels on a per microsphere basis and offer greater detection sensitivity (important in low-flow studies), (2) lower levels of Tween thereby reducing potential for adverse allergic reactions and (3) this new formulation is stable to autoclaving; NAM are provided autoclaved (important advantage for long-term survival studies). NAM have recently been used in chronic studies to measure temporal changes in blood flow at the level of the microcirculation [23]; in this setting

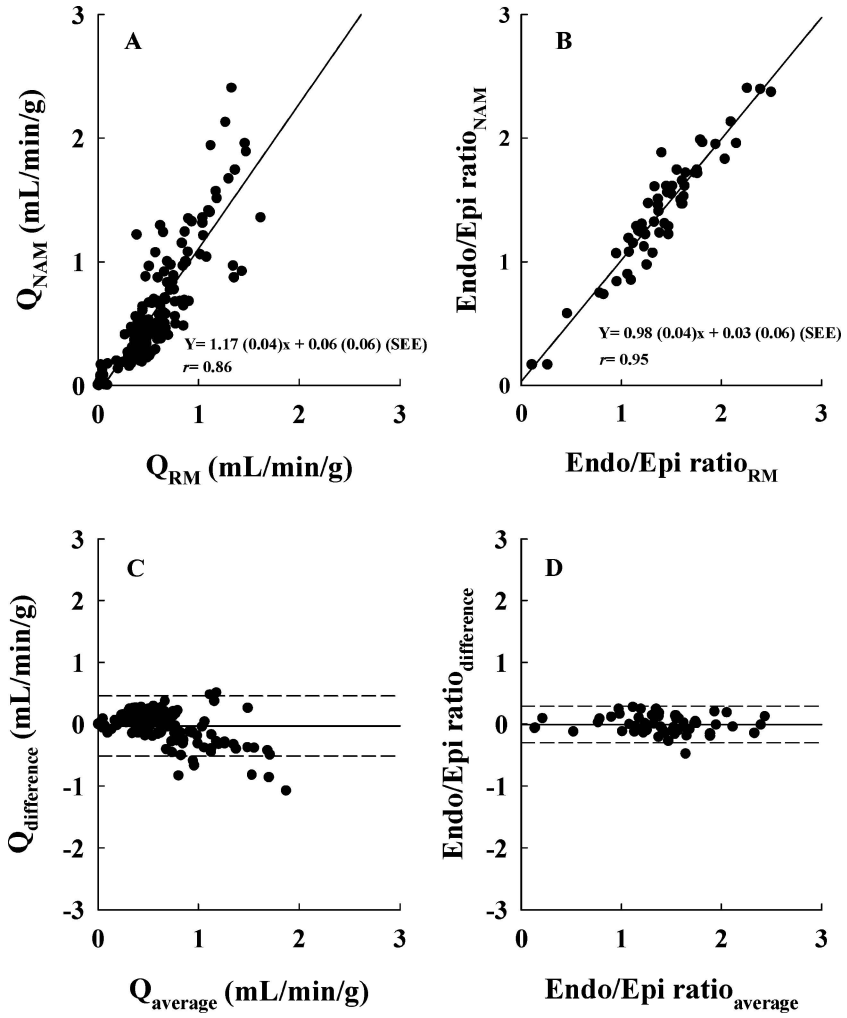

Fig. 3. *Intermethod comparison of blood flow and endo/epicardial blood flow ratios for myocardial tissue samples. A: blood flow and B: endo/epicardial blood flow ratio measured with NAM in relation to co-injected RM. Regression lines and r values were computed from all data; each data point represents a single myocardial tissue sample. For the endo/epicardial tissue ratios each data point represents the calculated ratio from two tissue samples in the same myocardial slice. Panels C and D illustrate intermethod differences (mean ± 2 SD indicated) between NAM and RM.*

generation of hazardous biological waste materials is not a predisposing factor.

Reinhardt and co-workers [9] validated the NAM technique for assessment of myocardial blood flow but they did not evaluate transmural distribution of blood flow which may not have been the same if the physical characters of NAM differed from RM. In order to use this new technology in our laboratory it was imperative to demonstrate that transmural distribution of blood flow was similar when measured by NAM or RM. The similarity of endo/epicardial blood flow ratios for NAM and RM provides further confirmation that NAM can be used to study transmural blood flow regulation in the heart and other organs.

In conclusion, results of this study in a large experimental model show that NAM accurately measure myocardial blood flow and its transmural distribution over a wide range. An additional advantage is the limited production of radioactive waste materials and availability of more labels. This technique should allow laboratories previously without access to RM to include measurements of organ blood flow in their studies.

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