Journal of Cardiovascular Magnetic Resonance (2007) **9,** 549–556

ISSN: 1097-6647 print / 1532-429X online
DOI: 10.1080/10976640601015177

informa
healthcare

A Combined Method for the Determination of Myocardial Perfusion in Experimental Animals Using Microspheres and CMR

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ABSTRACT

A convenient method is introduced for myocardial perfusion research by combining multislice short-axis cine cardiovascular magnetic resonance (MSA-CMR) and colored microspheres (CM). In canine control-ischemia-reperfusion ($n = 11$), Cardiac output (CO) was measured using MSA-CMR (CO_{MSA}), Phase Contrast CMR (PC-CMR, CO_{PC}) and CM (from reference blood samples, CO_{μ}) in 3 experimental periods per animal. CO_{μ} significantly and systematically overestimated CO_{MSA} (median deviation: 291 mL/min, $p < 0.01$), while there was excellent agreement between PC-CMR and MSA-CMR without significant over- or underestimation (median deviation: -14 ml/min, $p = NS$). In quantitative myocardial perfusion assay by CM, CMR should be used instead of reference blood sampling. This should improve the accuracy of absolute regional perfusion measurement.

INTRODUCTION

Cardiovascular magnetic resonance (CMR) is capable of the detection of both myocardial perfusion and function (1–3). CMR has become the "gold standard" in cardiac output (CO) measurements, in the determination of ventricular volumes (4–13). Cine CMR is also capable of high-resolution assessment of regional wall motion (14, 15). In an animal research setting, microspheres (colored, fluorescent or radioactive) have been considered the "gold standard" for the determination of regional myocardial perfusion (16, 17). The largest contribution to inaccuracy in this method results from the need for a reference blood sample for the purpose of determining CO, which is needed to calculate regional perfusion values. To date, the only method suggested to circumvent this problem has been the direct measurement of absolute blood flow somewhere along the circulation by means of a flowprobe. This is a complicated and invasive method, plus the flowprobe may induce image artifacts (16).

The aim of our present study was to introduce a new method for the determination of regional myocardial perfusion by combining CMR measurement of CO with the systemic administration of colored microspheres (CM). This method should improve the accuracy of regional myocardial perfusion quantification.

In a canine model of control-ischemia-reperfusion, repeat measurements of CO were carried out using multislice short-axis (MSA-CMR) or Phase Contrast CMR (PC-CMR), as well as using colored CM (from reference blood samples, CO_{μ}) in each of the 3 experimental periods. The purpose of the transient ischemia and reperfusion periods was to induce changes in CO to investigate the accuracy of measurements in a wider range of CO values.

Received 2 February 2006; accepted 15 August 2006.
Keywords: Myocardial Perfusion, Cine CMR, Phase Contrast CMR, Microspheres, Myocardial Ischemia.
This work was presented in part at the Annual Meeting of the NASCI, October 2003 Dallas, Texas, where it was the joint winner of the 3rd Annual Young Investigator Award Sponsored by the Council on Cardiovascular Radiology & Interventions, American Heart Association. We would like to acknowledge partial support for this work by grants R44 HL58285 (P.S.) and RO1 HL63340 (G.A.E.).
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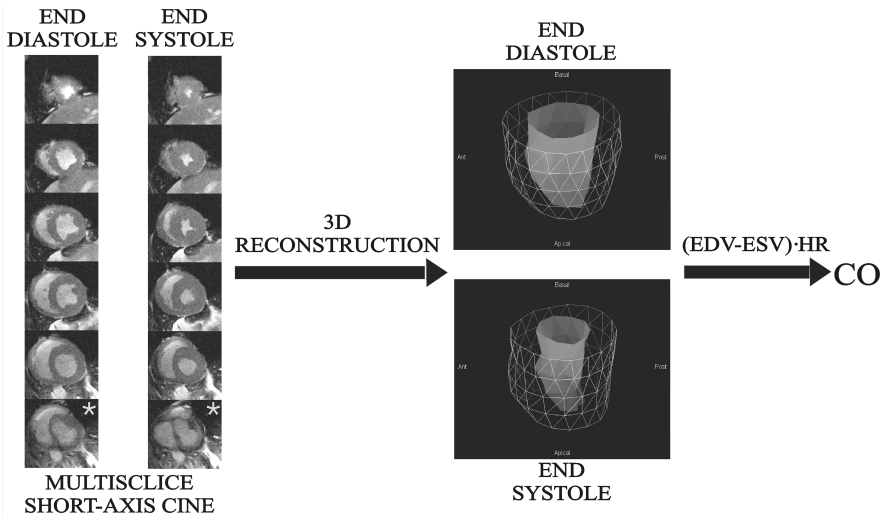

Figure 1. Two sets of multislice short-axis SSFP images (end-diastolic and end-systolic) are shown, used for the purpose of measuring flow in the ascending aorta, i.e., the stroke volume (SV). The difference between the reconstructed end-diastolic (EDV) and end-systolic volumes (ESV) yields SV (HR = heart rate). *the basalmost slice was omitted from the SV calculation, as most of the cavity in it belonged to the atrium and the aortic root.

METHODS

Canine model

This protocol was approved by the University of Alabama at Birmingham, Institutional Animal Care and Use Committee (UAB IACUC) and procedures were in accordance with institutional guidelines. Eleven male dogs weighing 18–20 kg (Marshall Farms, North Rose, NY) were anaesthetized using a mixture of ketamine (5.0 mg/kg; Lloyd, Shenandoah, IA) and diazepam (0.5 mg/kg; Hospira, Lake Forest, IL) I.V. Following intubation, anesthesia was maintained by continuous administration of isoflurane (2.5–3 %V/V; Minrad, Bethlehem, PA) and fentanyl (50–100 ug I.V.; Abbott Laboratories, North Chicago, IL) was administered every 30 minutes for analgesia.

One of the femoral arteries was cannulated, and a long catheter was inserted into the abdominal aorta to collect blood samples during CM injections. The other femoral artery was cannulated to monitor blood pressure. ECG was continuously recorded. A left side thoracotomy was performed. To induce acute ischemia, the left anterior descending coronary artery was surgically isolated after the first diagonal branch, and a snare was placed around it for later occlusion. The aperture of the thoracotomy was closed to restore a near-normal anatomical situation. To ascertain whether the vessel occlusion was effective, a 20 s zero-flow ischemia test was performed, monitored by ECG.

CMR

A 1.5 T, GE Signa CV/i whole-body scanner was used for imaging. Gas anesthesia was maintained during the CMR sessions. Following the CMR session, the dogs, still anesthetized, were sacrificed using pentobarbital and potassium chloride injections.

A multislice short-axis cine CMR (MSA-CMR) image set of the heart was generated using a steady-state free precession (SSFP) cine sequence (2, 18). Five or six slices were acquired covering the LV, using the following imaging parameters: FOV = 300 mm, matrix = 224 · 224, slice thickness = 8 mm, spacing = 2 mm, flip angle = 25°, phases = 20/cardiac cycle, TE = 1.83 ms, TR = 4.34 ms, views per segment = 8, NEX = 1, temporal resolution = 20 – 30 ms depending on heart rate. Mean ± SD signal intensity in myocardium and blood was 99.4 ± 19.1 and 212.0 ± 21.4 SIU, respectively (for reference, the background noise was 19.4 ± 4.6 SIU.)

Image analysis was carried out using MASS 5.0 software (Medis, Leiden, The Netherlands). Endocardial contours were traced manually in all slices. The 3D reconstruction of the whole left ventricle was obtained in this manner (Fig. 1). The difference between end-diastolic and end-systolic volumes yielded the stroke volume (SV). The heart rate was recorded during the experiment and also used for CO_{MSA} calculations.

Phase Contrast CMR (PC-CMR) images of the ascending aortic blood-flow were collected with the following parameters: FOV = 300–340 mm, matrix = 256 · 128, slice thickness = 10 mm, flip angle = 15°, phases = 20/cardiac cycle, TE = 3.2 ms, TR = 7.8 ms, views per segment = 4, NEX = 1, temporal resolution: 20 – 30 ms depending on heart rate, VENC = 200 cm/s. Maxwell correction was not used in our phase-contrast velocity mapping method (19) We did not use background correction as it is difficult to carry out in the mediastinum due to large vessels and air in the vicinity of the ascending aorta (8). Images were analyzed and SV was determined using the software FLOW (Medis), and CO_{PC} was calculated (Fig. 2). The product of the heart rate and the SV, obtained by either of the above CMR methods, yielded CO. The analysis of MSA-CMR was performed independently of

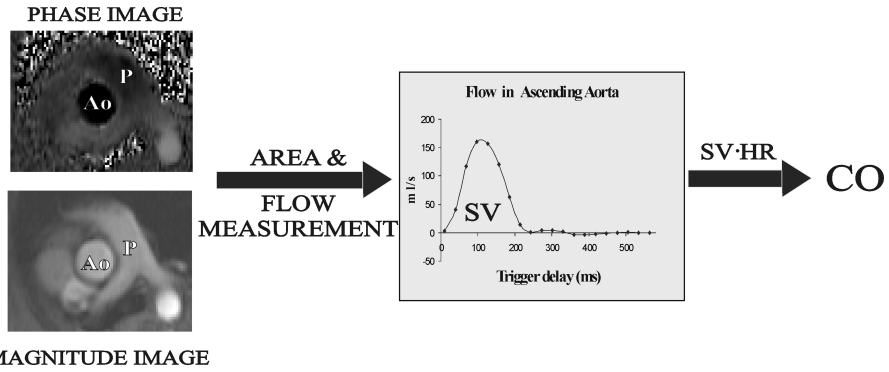

Figure 2. Phase contrast fast gradient echo velocity mapping used for the purpose of measuring flow in the ascending aorta (Ao), i.e., the stroke volume (SV). The pulmonary artery is denoted with P. The area under the flow velocity curve yields SV (HR = heart rate).

the PC-CMR analysis and blinded to the results of the other method.

CMR protocol

By means of scout images the true long axis of the left ventricle (LV) was determined. MSA-CMR images were positioned perpendicular to the long axis of the heart. PC-CMR slices were positioned perpendicular to the ascending aorta, at the level of the pulmonary artery. During subsequent breath holds, both types of CMR acquisitions were collected in each of the experimental periods. The first image set was obtained before inducing ischemia (control), the second set during the 30 minute LAD occlusion (ischemia), and the third set during reperfusion (2 dogs were lost during ischemia, and one during early reperfusion). In each of the periods, at the time of PC-CMR acquisition, different colors of CM were administered, and reference blood samples were withdrawn to determine CO_μ (Equation 1 below) according to the method of Kowallik et al. (20).

Microspheres

Three million CM, 15 ± 0.1 μm in diameter (DyeTrak, Triton Technology, Inc., San Diego, California, USA) were injected into the left atrium through a Tygon catheter during each experimental period. The catheter was flushed with at least 10 mL of a room temperature saline solution. Before injection, the CMs were ultrasonicated and vortex-agitated for effective dispersal. To calculate the actual blood flow in units of mL/min, a blood sample was collected during the injection of each CM set, using a catheter introduced into the ascending aorta through the femoral artery. Withdrawal of blood started 10 s before the injection of the CM and continued for 100 s at a rate of 0.1 mL/s. The blood samples were divided into 2.5 mL portions each and were digested in a glass tube with a 4 M KOH solution containing 0.05% "Tween 80" at 60°C overnight. After digestion, all samples were filtered through a glass filter equipped with a polyethylene filter membrane of 10 μm pore size to remove CM from the solution.

The dyes were dissolved in 300 μl dimethyl-formamide and the samples were centrifuged for 5 minutes at 14,000 rpm in an Eppendorf microcentrifuge to remove the spheres and the filter membrane from the solution. The samples were analyzed using a Hewlett-Packard 8452A Spectrophotometer (Houston, TX). Spectral analysis was carried out in the range of 320–820 nm with the method of Multicurve Analysis (MCA). The data from the blood samples were used to calculate CO_μ (i.e., cardiac output determined from microspheres) using the following equation:

$$CO_\mu = (F_{reference} \cdot N_{injected})/N_{reference} \quad [1]$$

where $F_{reference}$ stands for the withdrawal rate of the reference blood sample (0.1 mL/s). $N_{injected}$ and $N_{reference}$ represent the number of microspheres injected into the left atrium and the number of microspheres detected in the reference blood sample, respectively.

Statistical analysis

Statistical analysis of data obtained was carried out using SigmaStat (version 2.03; SPSS, Inc, Chicago, IL). To compare CO_{MSA} among the 3 experimental periods, Kruskal-Wallis Analysis of Variance on Ranks was used. To compare overall errors of CO_μ and CO_{PC} (relative to CO_{MSA}, the gold standard for CO), data were pooled from all experimental periods. The data did not pass the normality and equal variance tests. Therefore, a non-parametric test, Mann-Whitney Rank Sum Test, was carried out on the pooled data from all 3 experimental periods (control, ischemia, reperfusion) to test significance, and median, and 25th and 75th percentiles (in parentheses), are shown. Additionally, the same analysis was then carried out on the data from each experimental period separately. A p value below 0.05 indicated statistical significance. Pearson's Product Moment Correlation analysis was carried out for CO_μ vs. CO_{MSA}, as well as for CO_{PC} vs. CO_{MSA}. A p value of less than 0.05 was accepted as statistically significant.

RESULTS

The MSA-CMR method is considered the cardiology gold standard for the determination of cardiac output (21). Thus, we have compared both the CO_μ and the CO_{PC} results to CO_{MSA}.

Median CO_{MSA} in the control period was 1909 mL/min (1393, 2382). During ischemia, the median CO_{MSA} decreased to 1563 mL/min (1340, 2273). Varying extents of ischemia (due to varying extent of collateralization in individual dogs) caused the large variability in CO values during ischemia among the animals. Not all animals experienced severe reduction of CO. During reperfusion, the median CO_{MSA} of 1980 mL/min (1536, 2715) was slightly higher than that in the control period. No significant differences (p = NS) were found among the 3 experimental periods.

Significant overall correlation was found between CO_μ and CO_{MSA} ($n = 28$, $R = 0.87$, $p < 0.01$) (Fig. 3, A). The CM method systematically overestimated the MSA-CMR method by a median value of 291 mL/min (81, 637) ($p < 0.001$). This median overestimation constitutes 15.6% of the median CO obtained by MSA-CMR itself. The linear regression line of all CO_μ measurements versus all CO_{MSA} measurements was $y = 1.17 \cdot x$ (Fig. 3A), indicating that the CM measurement systematically overestimated CO. Interestingly, the deviation of the CO_μ values from the line of identity (i.e., from CO_{MSA}) was greater at CO_{MSA} values above 1500 mL/min, than it was at lower CO values. The unreliability (scatter) of the CO_μ values was also more pronounced at the higher range of CO values. These facts suggest that measurements at higher and higher CO values may be less and less reliable.

We have also found significant overall correlation between the CO_{PC} and CO_{MSA} ($n = 28$, $R = 0.95$, $p < 0.01$). (Fig. 3, B) The PC-CMR method showed almost identical CO values with those determined by the MSA-CMR method. The statistical analysis showed a median underestimation of CO of only −14 mL/min (−147, 161) (p = NS) by PC-CMR vs. MSA-CMR. This median overestimation constitutes 0.8% of the median CO_{MSA}. The linear regression line of all CO_{PC} measurements versus all CO_{MSA} measurements was $y = 1.003 \cdot x$ (Fig. 3, B). Thus we have confirmed the excellent agreement between CO determined by PC-CMRI and MSA-CMR.

The same trends were observed when analyzing data from each experimental period separately. Figure 4 shows linear regression analyses of CO_μ and CO_{PC}, both vs. CO_{MSA}, in the control, ischemia and reperfusion periods. Table 1 shows the slopes, correlation coefficients and median overestimations for each period as well as for the data pooled from all 3 periods (overall results).

Figure 5 shows Bland-Altman plots both for the bias observed with microspheres (Fig. 5, A) and with phase contrast CMR (Fig. 5, B), and both vs. short axis multislice CMR. Box plots of the overall deviations from CO_{MSA} of the CO_μ and CO_{PC} values are shown in Figure 6. Deviation values for CO_μ were significantly higher than those for CO_{PC} ($p < 0.001$), indicating again that CM significantly overestimated CO compared to either CMR method. When data from each of the three experimental periods were analyzed separately, similar results were obtained. Median deviations for CO_μ were 352 (27, 659), 375 (182, 725) and 197 (111, 514) mL/min in the control, ischemia and reperfusion periods, respectively. In all three periods the overestimation was significant with p values 0.01, smaller than 0.001, and 0.01, respectively. Median deviations for CO_{PC} were −31 (−130, 215), 47 (1, 144) and −129 (−189, 37) mL/min, for the same periods, respectively (p = NS for all 3 periods).

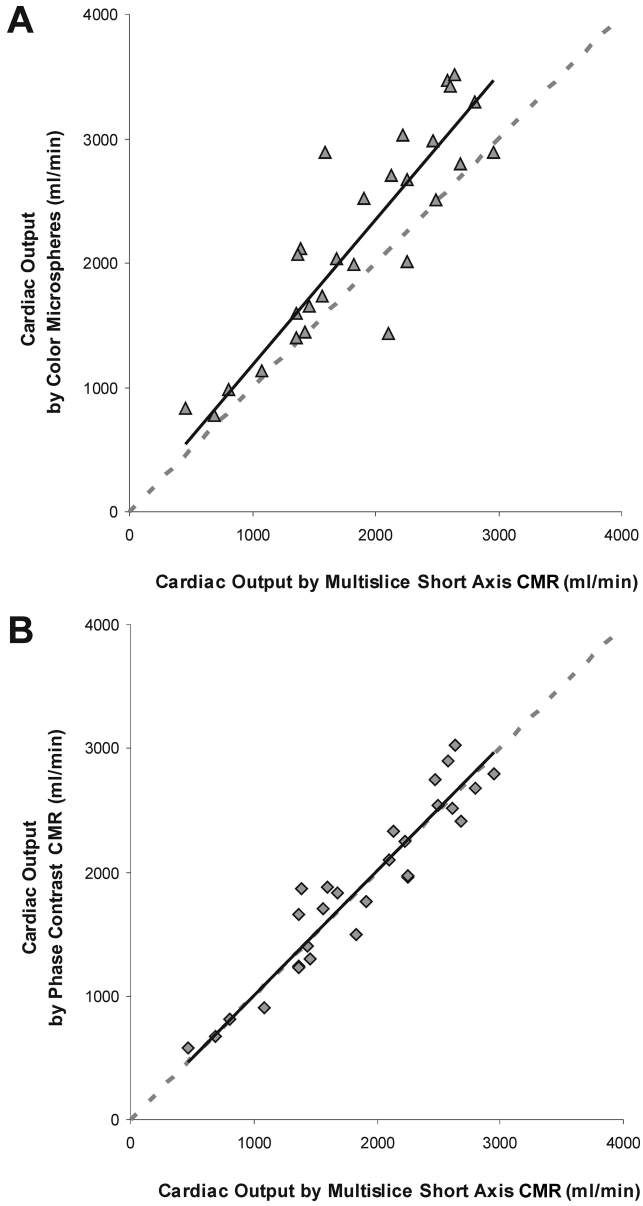

Figure 3. Correlation plots of CO as measured by CM (CO_μ, triangles in Fig. 3, A) or PC-CMR (CO_{PC}, diamonds in Fig. 3, B) vs. the gold standard, i.e., MSA-CMR (CO_{MSA}). Dotted and solid lines indicate the line of identity and the line of linear regression, respectively. The correlation between CO_{PC} and CO_{MSA} is highly significant ($R = 0.95$, $p < 0.01$) and the linear regression line is close to the line of identity ($y = 1.003 \cdot x$) (Fig. 3,B). The correlation between CO_μ and CO_{MSA} is weaker, but still significant ($R = 0.87$, $p < 0.01$) (Fig. 3, A). The slope of the latter regression line ($y = 1.17 \cdot x$), however, is substantially larger than 1.0, suggesting that the CM measurement systematically overestimates CO_{MSA}, especially in the high range of CO (>1500 mL/min) ($n = 28$).

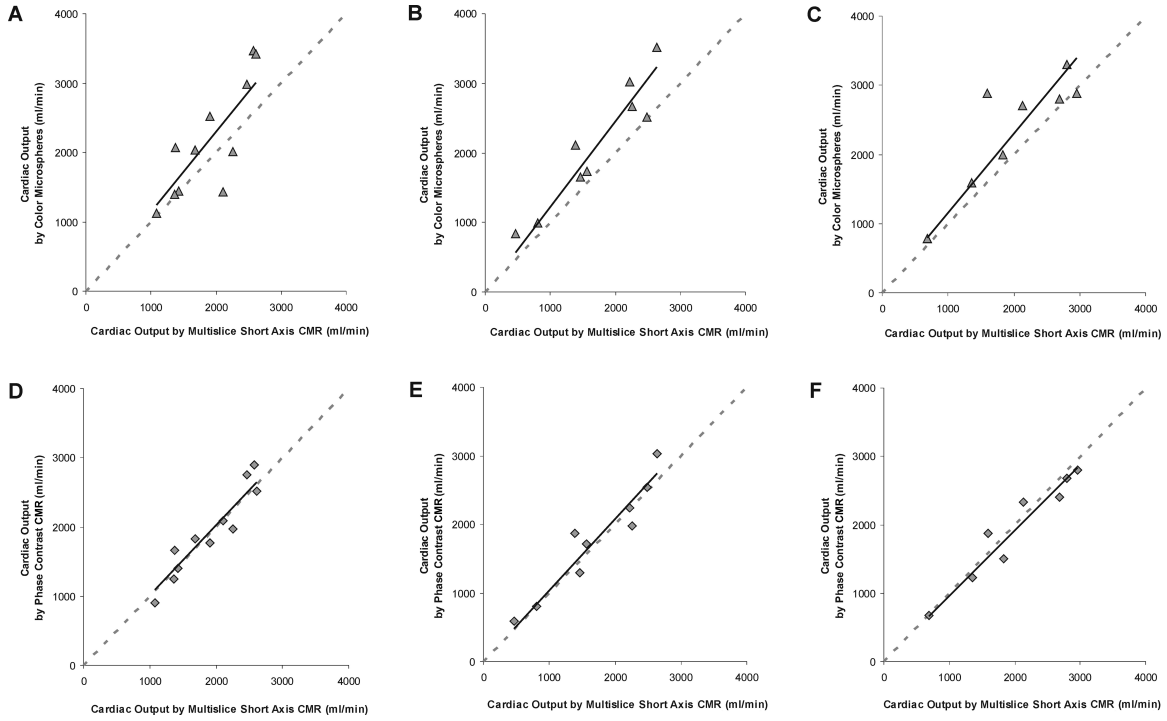

Figure 4. Correlation plots of CO in each experimental period as measured by CM (CO_{μ}, triangles in Figs. 4, A, B, and C) or PC-CMR (CO_{PC}, diamonds in Figs. 4, D, E and F) vs. the gold standard, i.e., MSA-CMR (CO_{MSA}). Experimental periods are shown from left to right in the following order: control, ischemia, reperfusion. Regression lines are solid, lines of identity are dashed. Slopes of regression lines and correlation coefficients are shown in Table 1.

DISCUSSION

We have shown that CO measurement using CMR is more reliable than with CMs. Although the differences in CO among the experimental periods (control, ischemia, reperfusion) were not significant, this could have been caused by the apparently large differences in baseline CO among the dogs. Similar trends were observed in all three experimental periods, as in the pooled data. Thus, our observation of the microsphere method overestimating the CMR methods appears to be present in both the presence and the absence of LV dysfunction.

Regional myocardial blood flow measurement using the CM method is a gold standard in animal experiments (20, 22-24). An accurate measurement of CO is necessary to determine absolute values of regional myocardial blood flow. This measurement, however, requires a reference blood sample to be taken from the aorta using a constant withdrawal rate (20). Our results show that the CO measurement using the CM method could be improved upon. The systematic error resulting from the use of CM to measure CO is probably due to loss of CMs in the course of withdrawal, digestion, and processing of blood samples. Therefore, the measured count in the sample is lower than the actual number of CM withdrawn. This potentially results in the overestimation of CO (Figs. 4A–C, 5A and 6). This error in the measurement is carried over to the calculation of the regional perfusion values:

$$F_{sample} = (N_{sample}/N_{injected}) \cdot CO_{\mu} \qquad [2]$$

where F_{sample} and N_{sample} are the regional myocardial blood flow and the number of CMs detected in the myocardial sample, respectively. Thus, from Equations 1 and 2 the following can

Table 1. Slopes of regression lines, correlation coefficients and median overestimations in each of the experimental periods, and overall.

	CM vs. MSA			PC vs. MSA		
	Slope	R	Median Overestimation	Slope	R	Median Overestimation
Control	1.16	0.82	352.3	1.01	0.94	−31.3
Ischemia	1.23	0.94	374.7	1.04	0.95	46.7
Reperfusion	1.15	0.83	197.5	0.96	0.96	−129.5
Overall	1.17	0.87	291.2	1.00	0.95	−14.1

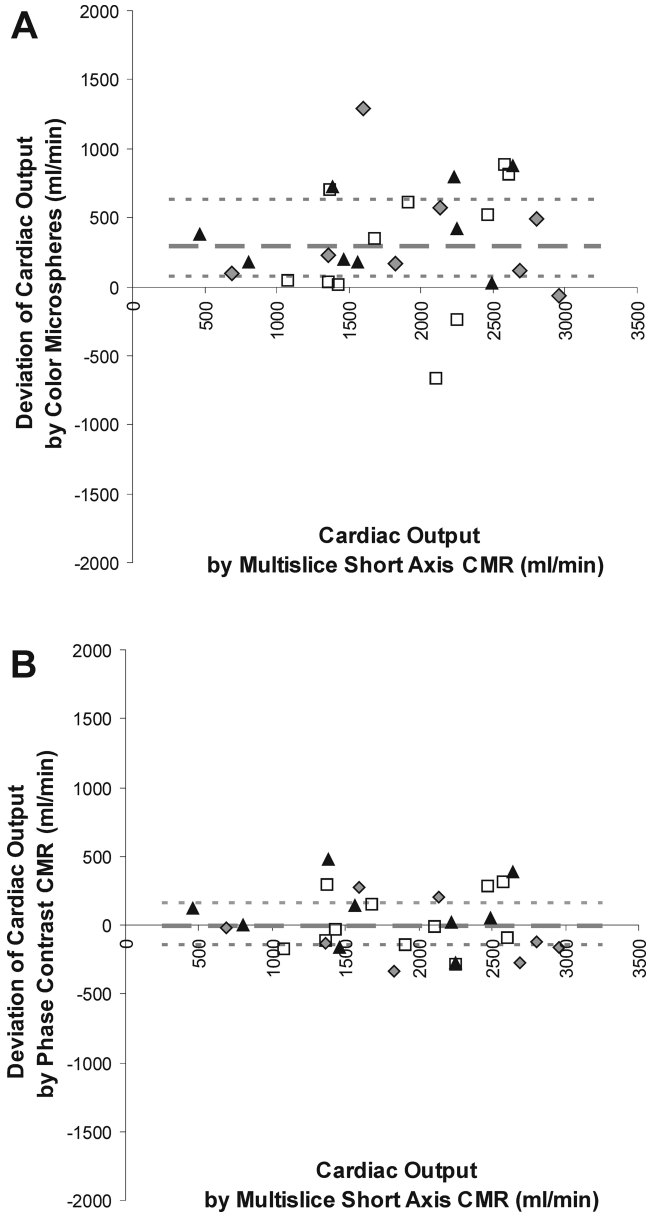

Figure 5. Bland-Altman plots for both the bias observed with microspheres (A) and with phase contrast CMR (B), both vs. short axis multislice CMR. The 3 experimental periods are denoted with different symbols (open squares = control, black triangles = ischemia, gray diamonds = reperfusion). Shown are overall median deviations (bias) as dashed gray lines, and 25th and 75th percentiles as dotted gray lines.

be derived:

$$F_{sample} = (N_{sample}/N_{injected})\cdot((F_{reference}\cdot N_{injected})/N_{reference}). \quad [3]$$

From which it follows that:

$$F_{sample} = (N_{sample} \cdot F_{reference})/N_{reference}. \quad [4]$$

Clearly, the accuracy of F_{sample} is dependent on the accuracy of $N_{reference}$. Unfortunately, however, $N_{reference}$ is unreliable because in the course of acquiring and processing blood samples, a number of factors can influence the eventual count of CMs found in the blood sample.

These factors are as follows. There is no information about the CM distribution profile at the flow front when traveling with blood flow in the descending aorta. The actual position of the sampling catheter within the great vessel (whether closer to the wall or to the center, or in a section with turbulent flow) may influence the number of spheres sampled. It is also possible that the catheter is inserted by accident into a smaller branch of the aorta. In the course of blood sample acquisition and processing, CMs may be lost. Microspheres may be trapped in the blood sampling pump which is equipped with long tubing. During digestion of the blood samples, some microspheres may be damaged, and during filtering some further CMs may be lost. There may also be errors associated with dissolving the dyes from the CMs to prepare samples for spectrophotometry.

By definition, according to Equation 1, the higher the CO, the smaller the number of CMs in the reference blood sample ($N_{reference}$). Thus, at the higher range of CO values, even small errors in blood sampling and processing may cause great distortions in calculating CO_{μ}, and these errors may cause even larger distortions in calculating regional perfusion values.

A few, but far from all, processing steps of myocardial samples are identical to those used to process blood samples. While errors caused by myocardial sample processing may compensate partially for errors in CO measurement from blood samples, the random nature of these errors makes this compensation nonuniform over the various samples in the left ventricle. The error in CO measurement, however, affects the perfusion measurement in each of the samples equally.

The method of obtaining the tissue samples, however, is fundamentally different. While reference blood samples are withdrawn using a long plastic catheter, tubing and a pump, myocardial samples are merely sectioned. It is also noteworthy that if CO and myocardial perfusion increase, there will be more microspheres in myocardial samples but less microspheres in reference blood samples than when CO and regional perfusion is decreased. Thus, the dynamic ranges of measurement for myocardium vs. blood sample behave in opposite ways. These differences lead to error types that do not cancel each other

CMR has become the "gold standard" method to determine CO (21). In animal research where CMR is used, this fact can be turned to an advantage by offering a solution to the above discussed problem. The aim of our current work was to introduce a new method, combining CM and CMR. Using such a combination, the regional myocardial blood flow measurement becomes simplified and more accurate. SSFP cine imaging (using retrospective gating and breathholding) is the most frequently used sequence to generate moving images of the beating heart to estimate ventricular volumes (2). One approach, MSA-MR generates multiple images along the short-axis of the heart to obtain whole-heart coverage (Fig. 1). Endocardial contours are then traced in the end-systolic and end-diastolic phases in all slices and a 3D reconstruction of the whole left ventricle

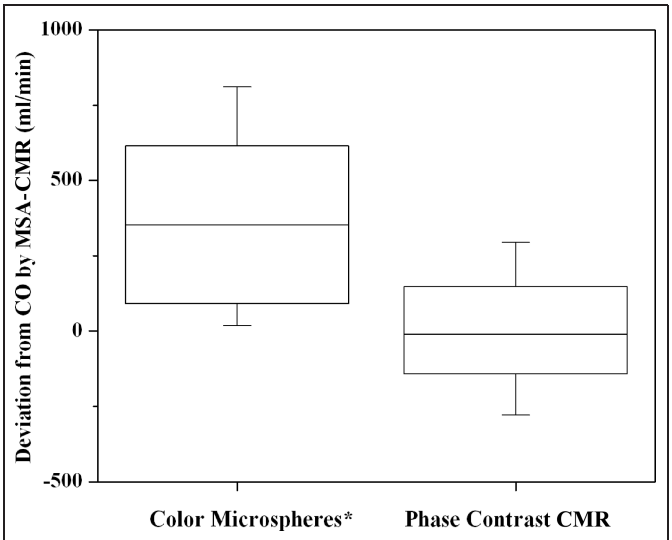

Figure 6. Median (line inside the box) and percentiles (10th & 90th, and 25th & 75th are presented as whisker and box boundary, respectively) of deviations of CO_{μ} values from the gold standard CO_{MSA} values (box of Color Microspheres) and of deviations of CO_{PC} values from CO_{MSA} values (box of Phase Contrast CMR). The asterisk (*) for the CM method denotes statistically significant difference from CO_{MSA}.

yields measurement of LV volumes. The difference between end-diastolic and end-systolic volumes yields the stroke volume (Fig. 1).

The advantages of MSA-CMR are that the irregular, tortuous shape of the left ventricle is taken into account and ventricular volumes are accurately measured and that, additionally, regional wall motion can also be quantified using these images. The disadvantages are partial volume effects, especially near the apex, leading to blurring of endocardial contours, the longer time needed for image acquisition and analysis, compared to PC-CMR, and the fact that in case of valvular dysfunction (e.g., aortic or mitral valve insufficiency) the measured stroke volume will be erroneous.

ECG-gated, segmented, PC-CMR is an established technique whereby through-plane velocities are measured in an imaging plane oriented typically perpendicular to the flow direction by encoding the spin velocity to the image phase (Fig. 2). Conventional phase-difference processing of the flow-encoded image data yields velocity images. Important advantages of PC-CMR are that acquisition of a single slice is sufficient, it is easy to evaluate, and that small errors in image angulation (i.e., when the imaging plane is not exactly perpendicular to the vessel of interest) (25) are compensated by the vessel cross sectional area, which ultimately yields very accurate measurements.

The present study has shown excellent correspondence between the MSA-CMR and PC-CMR methods, confirming that in healthy animals or in transient ischemic models, either of these methods may be used in conjunction with microspheres for absolute regional flow quantification. Thus, we propose that the CO value measured by MSA-CMR or PC-CMR, synchronized to CM administration (or, in a similar manner to administration of radioactive or fluorescent microspheres), be used for the calculation of regional perfusion values.

$$F_{sample} = (N_{sample}/N_{injected}) \cdot CO_{CMR.} \quad [5]$$

Using CMR, when available, determination of cardiac output is more consistent, noninvasive and simplifies the present routine used in research laboratories. The method eliminates the need for reference blood sample withdrawal and avoids the complicated laboratory work needed to process these samples. In addition, it provides more accurate absolute values of regional perfusion. Regional wall motion and wall thickening values calculated from the MSA-CMR may also serve as an internal quality control for the detection of myocardial ischemia when such information is desired.

Study limitations

Our study relied on two fundamentally different CMR methods for reference CO measurements, but no CMR-independent method was used to validate results (e.g., thermodilution). While CMR results can be accepted as a gold standard measure of cardiac output, relying on 5–6 short axis slices with a thick slice thickness of 8 mm and a slice-gap of 2 mm is suboptimal. The rationale for using 8 mm thick slices in this study was to save time without degrading the signal-to-noise ratio.

The methods in this study were not designed to determine the specific possible causes of inaccuracy in CO measurement using microspheres, although the systematic deviation from CMR measurements suggests a problem in blood sample withdrawal and processing.

Our novel method does not eliminate any errors caused by possible loss of microspheres (colored or fluorescent) during the processing of myocardial samples, which would lead to the underestimation of regional perfusion. This type of error, however, is a random error, affecting individual samples to varying extent. In the case of radioactive microspheres, however, where practically no postprocessing is required, this would not be an issue.

Another source of error which is not eliminated by our method could be inaccuracies of the number of microspheres administered. Microspheres have a tendency to settle in their vials (hence the need for ultrasonic agitation), clump, and stick to tubing, syringes, or stopcocks. In our study, microspheres were administered using a short, 10 cm long atrial catheter attached via a stop-cock to two syringes, one with the microspheres, and another with the saline flush. Although it was visually confirmed that all microspheres had been flushed, some might have been trapped in the above devices. This may have contributed to the overestimation of CO by the microsphere method.

The inaccuracies in regional blood flow determination caused by overestimating the CO when using microspheres is not directly proven by our data. From Equation 2, however, it may be inferred that the overestimation of CO by microspheres leads to systematic overestimation of regional perfusion values for all samples.

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