Technique for Using Video Microscopy and Indicator Dilution for Repeated Measurements of Cardiac Output in Small Animals

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Background: The authors developed an indicator dilution technique for small animals to repeatedly determine cardiac output and blood volume without cardiac instrumentation or blood sampling.

Methods: Observations were made in the hamster (N = 32, 70 mg/kg pentobarbital) cremaster using in vivo fluorescence videomicroscopy. Fluorescein isothiocyanate–conjugated bovine serum albumin (10 mg/ml) was injected as a bolus dose (right jugular) while video recording the light intensity in a 20-μm arteriole (intensified charge-coupled device [CCD] camera at fixed gain). The intensity signal was analyzed over time (background subtracted) and calibrated to the dye concentration. The ex vivo calibration was performed using a constant optical path length (20 μm) and a range of dye and hematocrit concentrations. In vivo tube hematocrit was determined using standard methods with fluorescently labeled erythrocytes. Thus, quenching of the fluorescence signal by hemoglobin was corrected for the calibration, and the plasma space in the arteriole was determined. The steady state dye concentration measured by the light intensity at 2 min was not different from the dye concentration found by direct spectrophotometric analysis of the plasma.

Results: Cardiac index was calculated as milliliters of blood per minute per kilogram body weight. The calculated cardiac index was 359 ± 18 ml · min⁻¹ · kg⁻¹, which is not different from the reported values for hamsters. Cardiac output was increased twofold when enough intravenous nitropusside or nitroglycerine was injected to decrease mean arterial pressure from 90 to 70 mmHg. Cardiac output was elevated during dobutamine infusion (16 μg · kg⁻¹ · min⁻¹) and decreased during esmolol infusion (50, 75, 100 μg · kg⁻¹ · min⁻¹). Blood volume determined from the steady state dye concentrations was 6.2 ± 0.5 ml/100 g body weight, within the normal range for hamsters.

Conclusions: Fluorescent dye dilution and video microscopy can be used to repeatedly determine cardiac output or blood volume in small animals.

MEASUREMENT of cardiac output (CO) is technically challenging using the usual methods of thermodilution,1–3 microspheres,4,5 or classical indicator dilution. In small laboratory animals such as hamsters or mice, the technical difficulty is greatly amplified. There are realistic concerns with the extensive cardiac instrumentation required using thermodilution because the catheters cannot be made small enough to work properly while having no direct effect on cardiac function.5 On the other hand, microspheres, or indicator dilution techniques, require whole-blood sampling of 0.5 ml or more for measurement, which amounts to 10–20% of the blood volume in a 100-g animal.5 Thus, repeat measurements in the same animal involve significant volume loss. There is an additional concern with repeat measurements using large microspheres (> 5 μm) that significant microcirculatory changes occur between measurements because of vessel occlusion by the large particles.4

With an increasing move toward the small animal models for reasons of both cost and genomic knowledge, and to interpret peripheral changes in conjunction with systemic state, we developed an indicator dilution method to determine CO and blood volume in small laboratory animals. This allows direct observation of an indicator passing through a 20-μm arteriole in an in vivo microvascular preparation. This modification is relatively noninvasive, does not require cardiac instrumentation, does not require blood sampling for the measurement, and can be used for repeated measurements in the same animals. Here we outline the method and its verification.

Materials and Methods

After obtaining approval from the University of Rochester School of Medicine and Dentistry, adult male Golden hamsters (HSD-Syr; age, 78 ± 2 days; weight, 122 ± 9 g [mean ± SD]; N = 32) were anesthetized with pentobarbital sodium (70 mg/kg intraperitoneally) and tracheostomized. Body temperature was maintained between 37 and 38°C. The following catheters were placed: a right jugular catheter (PE50, drawn to a 200-μm tip) for injection of fluorescein isothiocyanate labeled erythrocytes or fluorescein isothiocyanate conjugated–bovine serum albumin (FITC-BSA; dye for CO), left carotid catheter for injection of microspheres, left femoral arterial catheter for withdrawal of the microspheres, and left femoral venous catheter for intravenous administration of nitropusside, nitroglycerine, dobutamine, or esmolol (dosages given in Results; catheters each PE50, drawn to approximately 100-μm tip). The carotid artery catheters were placed only in studies with microspheres. The right cremaster (n = 16 animals) or left cheek pouch (n = 16 animals) was prepared for in vivo microcirculatory observations.7 The preparation was continuously
superfused with bicarbonate-buffered saline containing 132 mm NaCl, 4.7 mm KCl, 2.0 mm CaCl₂, 1.2 mm MgSO₄, and 20 mm NaHCO₃ (equilibrated with gas containing 5% CO₂ and 95% N₂ gas, pH 7.4 at 34°C). All chemicals were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO) unless otherwise noted.

During a 60-min stabilization period, arteriolar tone was verified by dilation to topically applied 10⁻⁴ M adenosine, and constriction to 5% O₂ added to the superfusate, and fluorescently labeled erythrocytes (labeled with substituted tetramethyl rhodamine isothiocyanate [XRITC]; Molecular Probes, Eugene, OR) were administered. The XRITC erythrocytes were labeled using an established protocol from cells obtained from donor animals (n = 10).

**In Vivo Data Acquisition**

The CO measurements were made using only 20-µm-diameter arterioles. The microcirculation was observed with transillumination using a modified Nikon upright microscope (Nikon, Tokyo, Japan) with a 25× objective. Video images were produced using a charge-coupled device (CCD) 72s video camera and a Gen/Sys II video intensifier (Dage-MTI, Michigan City, IN), using a fixed gain setting. The erythrocyte velocity and flux were determined (XRITC cells) using a Chroma G1A filter set (Chroma, Brattleboro, VT) as previously described. Erythrocyte flux and velocity were used to calculate the tube hematocrit in the 20-µm arteriole and its inverse, plasma space. The hematocrit was required for more accurate calibration of the fluorescent indicator from light intensity to dye concentration because of quenching of the light signal by hemoglobin. The plasma space is the volume of distribution for the fluorescent indicator within the vessel chosen for study. The excitation–emission filters were switched to a Chroma B1E for fluorescence detection, and the intensified camera system was placed on fixed gain. This same gain setting was chosen for the *ex vivo* calibration. A small rectangular region (5 × 20 µm) in the centerline of the image of a 20-µm arteriole was selected, and the background light intensity was determined. A bolus dose of 50 or 100 µl of 10 mg/ml FITC-BSA was injected via the right jugular vein. The average light intensity within the rectangular region was continuously measured over a 2-min period postbolus injection.

**Ex Vivo Calibration of Dye Concentration**

The light intensity caused by the fluorescent tracer was converted to dye concentration (mg/ml plasma) using calibration equations defined by an ex vivo calibration system. Microslides with a 20-µm optical path length (#51447; Friedrich & Dimmock, Millville, NJ) were chosen so that the same optical path was used for the *in vivo* measurement and ex vivo calibration. Microslides were filled with a range of FITC-BSA (0, 0.01, 0.05, 0.1, and 0.15 mg/ml) and a range of hematocrit values (0, 1, 5, 10, 20% vol/vol) diluted in hamster plasma, for a total of 25 microslides per calibration. Each microslide was video-recorded using the same optical objective and optical path, with a fixed gain intensifier–camera setting and the same camera system as for the *in vivo* data. The average light intensity (background subtracted) was determined within a user-declared region of interest (5 × 20 µm, calibrated actual area) at three locations within each microslide; the three measurements were averaged. The calibration curve was constructed from the average light intensity as a function of FITC-BSA concentration; one curve was constructed per hematocrit. The *in vivo* light intensity was converted to dye concentration from the appropriate calibration curve by matching the tube hematocrit *in vivo* to the hematocrit of the microslide. From this, a relation between dye concentration over time was obtained.

**Analysis of the Indicator Dilution Curve and Calculation of Cardiac Output**

Fluorescent dye was injected into the jugular vein, and the intensity in a 20-µm arteriole was measured during the first pass. Intensity was converted to concentration according to the calibration described above. The area under the curve of dye concentration passing the measurement site (in milligrams per liter plasma per second) was used to calculate the CO.

The area was calculated according to the methods described by Linton et al. This method uses a log normal curve to fit the concentration curve and define the total area of the first pass of the dye during high or low CO during the expected conditions of recirculation of dye. The raw data are best fit to this equation, and the area is calculated from the best-fit curve.

Cardiac output (l/min) was calculated from the amount of dye injected (milligrams; I), 50 or 100 µl of 10 mg/ml, the plasma space available for dye distribution (1 - hematocrit), and the area under the curve of the first pass of the dye (mg · l⁻¹ · s⁻¹) measured in the arteriole (A) as defined in equation 1:

$$ I \times 60 \over A \times (1 - Hct) $$

**Blood Volume Measurement**

Blood volume was calculated from the steady state dye concentration extrapolated back to time zero. This could only be determined from indicator dilution measurements, and not microsphere measurements, because it required steady state concentrations. The calculated blood volume was normalized to the weight of the animal (as milliliters of blood per gram body weight) and compared with the published values for hamsters.
Verification of Plasma Dye Concentration by Spectroscopy

After determination of CO by indicator dilution, five animals were immediately exanguinated by cardiac puncture 2–3 min after FITC-BSA bolus injection. The whole blood was centrifuged, and the dye concentration in the plasma sample was measured by spectrophotometry, calibrated against a known dye concentration dissolved in hamster plasma. This was a direct measure of the total dye concentration in the plasma in vivo at steady state. The spectrophotometric value was compared with the dye concentration calculated from the light intensity in the arteriole 2 min after bolus injection.

Cardiac Output Measured Using the Microsphere Technique

This modified indicator dilution technique was compared with the microsphere method in both paired and unpaired measurements. We used 15-μm-diameter fluorescently labeled (tetramethyl rhodamine isothiocyanate) microspheres (Transduction Laboratories, Lexington, KY). For the unpaired measurements, the animals were instrumented with a left carotid catheter placed in the left ventricle and a left femoral arterial line for sample withdrawal. The pump (500 μl/min) was started 10 s before bolus injection of microspheres (50 μl, 10⁶ microspheres/ml) and for 1 min after injection (total of 580 μl withdrawn). The ratio of microspheres per erythrocyte was determined by flow cytometry (University of Rochester), counting a total of 10⁶ erythrocytes per sample. Microsphere totals were likewise determined by sampling from the injection catheter; these totals ranged between 1 and 5 × 10⁵ microspheres/ml. Systemic hematocrit was determined by toe clip, from which the total concentration of microspheres in the blood sample was calculated. CO was calculated from the total microspheres injected, I (50 μl of 10⁵ microspheres/ml), the concentration of microspheres in the blood sample, M, and the pump withdrawal rate, R: CO = I/(M × R). There was no period of stabilization for these measurements; the CO was determined immediately after carotid catheter placement. For paired measurements, the animal was additionally instrumented with a jugular venous catheter, and the check pouch was prepared for microcirculatory observations. A 30- to 40-min stabilization period followed injection of the fluorescently labeled cells. Thus, the total time between carotid catheter placement into the left ventricle and determination of CO was approximately 60-90 min. The indicator dilution technique was performed first (in duplicate, 10 min apart), followed by the microsphere technique. Using flow cytometry, the microsphere fraction was determined independent of the rhodamine-labeled erythrocytes.

Statistics

Cardiac index (CI) was calculated as the CO divided by the weight of the animal (ml · min⁻¹ · kg⁻¹). Values are reported as the mean ± SEM. Comparisons were made by t test between groups. Repeated measurements were evaluated by analysis of variance for repeated measures. All differences were significant at P < 0.05.¹¹

Results

The image of fluorescent dye passing through a 20-μm arteriole after the bolus injection of FITC-BSA is shown in figure 1. The small rectangle indicates the region of analysis. Light intensity over time for a control CO measurement is shown in figure 2. The conversion from light intensity to plasma dye concentration was performed.
using the ex vivo calibration shown in figure 3. Importantly, this family of calibration curves is specific to the camera-intensifier system and to the gain setting.

The resulting dye concentration over time is given in figure 4 for conditions of high CO with dobutamine (fig. 4A) or low CO with esmolol (fig. 4B). The area of the first passage was used in the CO calculation (equation 1). The specific input parameters to calculate CI for figure 4A are: I = 100 μl of 10 mg/ml FITC-BSA = 1 mg dye; A = 0.586 mg · ml⁻¹ · s⁻¹; (1 - Hct) = (1 - 0.02); and the animal weight = 131 g. For figure 4B, these values are: I = 100 μl of 10 mg/ml FITC-BSA = 1 mg dye; A = 11.3 mg · ml⁻¹ · s⁻¹; (1 - Hct) = (1 - 0.07); and animal weight = 123 g. Note that this means the microvessel hematocrit for these experiments was 2 and 7%, which is common in all microvessels, despite the fact that the systemic hematocrit is much higher (for hamsters, 55%).

In paired samples, the steady state plasma concentration of FITC-BSA was not different by spectrophotometry sampled at 2–3 min after bolus injection, compared with values obtained from the indicator dilution method using the light intensity measured at 2 min (fig. 5).

Cardiac index was determined using the microsphere method for comparison to the indicator dilution method. Figure 6A shows unpaired estimates of CI, where the microsphere method and indicator dilution were used in different animals. CI was not different by these two techniques in the unpaired comparison. Paired estimates are also shown in figure 6A, where the two methods were used in the same animal. The indicator dilution method was always used first because of the likelihood of the 15-μm microspheres interfering with microvascular flow, and because 0.5 ml (10% blood volume) was removed during the microsphere measurement. We first compared CI by indicator dilution in animals that were instrumented versus not instrumented for the microsphere method (compare the open bars of fig. 6A). For control animals (no inotropic agents given), values for CI by indicator dilution are significantly lower in the paired estimates (279 ± 15 ml · min⁻¹ · kg⁻¹) compared with the unpaired estimates (356 ± 20 ml · min⁻¹ · kg⁻¹) (compare the open bars). We consider this is likely because of the instrumentation—the presence of a catheter in the left ventricle for 60–90 min before CI determination. Importantly, there was no difference, on average, between CI determined by the two methods for the group that was paired (indicator dilution vs. microsphere; fig. 6B). For one animal, CI decreased by half between measurements with indicator dilution and with microspheres. For the paired control determinations, heart rate (305 ± 8 beats/min) and mean arterial pressure (183 ± 4 mmHg) did not change between the two measurements.

Importantly, also at low CI induced by 75 μg · kg⁻¹ · min⁻¹ esmolol (n = 6), CI was not different when measured by these two techniques (fig. 6). Likewise, neither heart rate (250 ± 6 beats/min) nor mean arterial pressure (152 ± 10 mmHg) changed between the two determinations of CI.

For comparison, CO was determined during intravenous (femoral vein) administration of sodium nitroprusside (20 ± 4 μg · kg⁻¹ · min⁻¹; n = 6), nitroglycerine (7 ± 3 μg · kg⁻¹ · min⁻¹; n = 6), dobutamine (16 ± 3 μg · kg⁻¹ · min⁻¹; n = 4), or esmolol (50 ± 12 μg · kg⁻¹ · min⁻¹; n = 4). CO was elevated with 20-min infusion of nitrovasodilators or dobutamine and was significantly decreased with 5-min infusion of esmolol (fig. 7).

The blood volume was determined using the steady state dye concentration. The blood volume was 8 ± 15 ml · kg⁻¹.
0.6 ml (n = 15), which when normalized was 6.2 ± 0.5% body weight (milliliters of blood per gram body weight) or, in standard form, 6.2 ± 0.5 ml/100 g body weight.

Discussion

In this study, we report a modification of an indicator dilution technique for measuring CO in small laboratory animals. We independently verified this technique in two ways: the spectrophotometric determination of the steady state plasma levels of the injected dye matches that determined by the indicator dilution technique, and, secondly, the microsphere technique for CO provides the same value as the indicator dilution technique. We found that both CO and blood volume measurements using indicator dilution were in keeping with the values reported for hamsters.13

Use of this technique requires a straightforward, and necessary, data acquisition and calibration sequence. Data acquisition and ex vivo calibration must be performed using the same camera-intensifier system at a specified gain setting and the same batch of FITC-BSA. The calibration is most sensitive to hemoglobin when vessel hematocrit is between 1 and 10% (approximately 15% difference), with less quenching between 10 and 20% hematocrit (approximately 5% difference). The range of in vivo vessel (tube) hematocrit values in this sized arteriole generally is between 5 and 20%, and the time-averaged tube hematocrit changes with the flow conditions.8,9,12 Thus, the most precise measurements of CO are made when the tube hematocrit is simultaneously determined.

The camera-intensifier gain setting must be set so the light intensity from initial peak of the dye passage does

Fig. 5. (A) A typical absorbance versus dye concentration curve, used to determine the dye concentration by spectrophotometry. Shown are the steady state plasma levels of dye (B) determined by indicator dilution (at 2 min after bolus injection) or by spectrophotometry (2–3 min after bolus injection). Indicator dilution measurements were calibrated from the ex vivo dye calibration method. FITC-BSA = fluorescein isothiocyanate–conjugated bovine serum albumin.
not exceed the maximum 8-bit gray scale (0–255 range) of the image capture board. We found that choosing two gain settings for the ex vivo calibration provides a range within which to optimally observe the dye passage in vivo for tissues of different thickness and optical clarity. The gain setting is chosen during the test dose, which also clears the dead space from the jugular catheter.

In our study, the paired CO determination using both the indicator dilution and microsphere techniques in the same animals provided a lower value for CO than in animals in which microspheres were not used. The lowered CO in the paired animals is very likely a result of the time these animals spent in stabilization of the microcirculatory preparation (necessary for the indicator dilution method) while the heart was instrumented (left ventricular catheter). The smallest catheter size possible was used that would remain in place and not fold back with the force of flow out of the left ventricle. However, the presence of the catheters for an additional hour significantly and detrimentally affected CO. This is precisely the adverse situation we are seeking to avoid through the use of the modified indicator dilution technique.

Our laboratories study peripheral-blood flow and vasoactive responses. For most studies of peripheral responses, the animal is in a constant depth of anesthesia (systemic steady state), and the microcirculation is verified to be in a steady state. Increasingly, more studies examine the impact of systemic changes on peripheral responses, e.g., studies of altered cardiac function or systemic inflammatory states. There are then two factors to consider: (1) the microcirculatory changes we wish to understand; and (2) the overriding systemic changes that will indirectly affect microcirculatory function. For these studies it is essential to define the systemic cardiovascular state to begin to understand direct versus indirect changes within the microcirculation. With the modified indicator dilution technique, it is now possible to determine CO and circulating blood volume multiple times in the same animal during several systemic states, or to verify a constant systemic cardiovascular status.

References