Journal of the American Association for Laboratory Animal Science
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Vol 54, No 6
November 2015
Pages 687–693

Measurement of Blood Volume in Adult Rhesus Macaques (*Macaca mulatta*)

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Most biomedical facilities that use rhesus macaques (*Macaca mulatta*) limit the amount of blood that may be collected for experimental purposes. These limits typically are expressed as a percentage of blood volume (BV), estimated by using a fixed ratio of blood (mL) per body weight (kg). BV estimation ratios vary widely among facilities and typically do not factor in variables known to influence BV in humans: sex, age, and body condition. We used indicator dilution methodology to determine the BV of 20 adult rhesus macaques (10 male, 10 female) that varied widely in body condition. We measured body composition by using dual-energy X-ray absorptiometry, weight, crown-to-rump length, and body condition score. Two indicators, FITC-labeled hydroxyethyl starch (FITC–HES) and radioiodinated rhesus serum albumin (^{125}I-RhSA), were injected simultaneously, followed by serial blood collection. Plasma volume at time 0 was determined by linear regression. BV was calculated from the plasma volume and Hct. We found that BV calculated by using FITC–HES was consistently lower than BV calculated by using ^{125}I-RhSA. Sex and age did not significantly affect BV. Percentage body fat was significantly associated with BV. Subjects categorized as having 'optimal' body condition score had 18% body fat and 62.1 mL/kg BV (by FITC–HES; 74.5 mL/kg by ^{125}I-RhSA). Each 1% increase in body fat corresponded to approximately 1 mL/kg decrease in BV. Body condition score correlated with the body fat percentage (R^2 = 0.7469). We provide an equation for calculating BV from weight and body condition score.

Abbreviations: BCS, body condition score; BV, blood volume; DXA, dual-energy X-ray absorptiometry; FITC–HES, FITC-labeled hydroxyethyl starch; ^{125}I-RhSA, radioiodinated rhesus serum albumin; PV, plasma volume

Rhesus macaques (*Macaca mulatta*) are a commonly used species in biomedical research due to their similarities in anatomy and physiology to humans. Many research protocols involve frequent blood sampling. Most institutions set limitations on blood sampling intended to prevent physiologic compromise that could result in subject morbidity or introduce experimental confounds. These blood draw limits are typically expressed as a percentage of total blood volume (BV) taken over a given time period. BV is estimated in terms of volume of blood (mL) per body weight (kg) or as a percentage of body weight. A 'rule of thumb' that is commonly applied at biomedical institutions is the '10%–10% Rule,' wherein the maximal sample volume allowed is 10% of the blood volume, and the BV is estimated as 10% of the body weight (or 100 mL/kg).[11,22,31] Although formulas or rules governing blood collection volumes vary widely among institutions (Figure 1), all appear to use an estimated BV that assumes a fixed body weight:blood volume ratio. Given the generalized use of formulas that estimate BV and the considerable variation of these formulas among institutions, a standardized approach for BV estimation that is based on scientific evidence is needed.

The practice of using body weight as a surrogate measure for BV has been the accepted standard practice for decades.[6,11,17] The only primary scientific publications that explored BV in rhesus macaques were early nuclear medicine studies conducted in the 1950s. A 1955 publication describes an experiment using radioactive iodinated human serum albumin as a tracer agent to determine BV in 20 "prepuberty [sic] rhesus monkeys, 10 males and 10 females." [3] Blood volumes in that study group ranged from 49 to 71 mL/kg, with a mean of 60.9 mL/kg. In 1958, the BV of 18 monkeys, 6 to 9 mo old, was determined by using Evans blue dye (T1824) to measure plasma volume and ^{32}P to tag cells to measure cell volume; these calculations yielded a mean BV of 54.0 mL/kg.[12] However, both of the cited studies[3,12] used juvenile animals, which may not be representative of mature, geriatric, or obese subjects currently used at biomedical research facilities. Moreover, the assumption that BV is a linear function of body weight is likely erroneous. Overwhelming evidence in the literature demonstrates that body condition (or degree of obesity) has profound effects on the BV per kilogram of body weight in humans.[9,10,14,18] As a result, researchers may be inadvertently causing subject morbidity or confounding their research outcomes by performing excessive blood draws in obese animals. Conversely, the possible underestimation of BV in lean macaques may unnecessarily constrain researchers, resulting in lower scientific yields from animal subjects. For these reasons, an update of the current industry standards for estimating BV and consequently for determining blood-draw limitations is needed.

Although BV is not routinely measured in nonhuman primates, it has great clinical relevance in human medical practice for the evaluation of blood loss, polycythemia, congestive heart failure, syncope, and various endocrine disorders. Many techniques have been developed to measure blood volume.[8,15,20] In 1980, the International Committee for Standardization in Haematology recommended the use of radioactive chromium within autologous RBC to measure RBC volume and of radioactive iodine bound to donor-derived human serum albumin to measure plasma volume (PV).[1] This classic dual-isotope method was considered to be the 'gold standard' for BV determina-

Received: 12 Jan 2015. Revision requested: 16 Feb 2015. Accepted: 05 Mar 2015.
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Primate center	Estimated blood volume (mL/kg)	Withdrawal limit (% of blood volume)	Maximal volume of blood that can be collected (mL/kg)	Collection period (d)
A	56	10%	5.6	14
B	56	12.5%	7	21
C	60	20%	12	30
D	60	20%	12	30
E	80	15%	12	28
F	100[a]	10%	10	14
G	100[a]	10%	10	42
H	100[a]	10%	10	28

[a]Indicates application of the 10%:10% rule.

Figure 1. The 2014 blood volume collection guidelines from each of the 8 National Primate Research Centers in the United States.

tion. However, this method is labor-intensive, prone to errors, and considered clinically impractical. Since then, alternative methods have been developed in which PV was measured by using radioiodinated human serum albumin whereas RBC volume was deduced from Hct to determine BV. This method compared favorably with the dual-isotope method, producing equivalent results in much less time.[7,20] Given the hazards and expense of using radioactive materials, alternative means of measuring BV have been described that use fluorescence or dyes as indicators.[2,8,24,30]

Current techniques used for measuring BV typically follow indicator-dilution methodology. The basic principle follows the Law of Conservation of Mass:

$$C_1V_1 = C_2V_2$$

for which a known volume (V_1) and concentration (C_1) of an indicator is injected into a system, and the concentration of the indicator is measured (C_2); one can then solve for the unknown volume (V_2). When this formula is applied to determine BV, the indicators dissolve in the plasma only and do not enter the RBC, so C_2 is the concentration of the indicator in the plasma and the calculated 'unknown volume' (V_2) is plasma volume (PV). The Hct, the ratio of RBC volume to BV, can be used to extrapolate BV from PV by using the following formula:

$$BV = \frac{PV}{(1 - Hct)}$$

An underlying assumption of this method is that the plasma compartment is a 'closed system,' or at least in a steady state. Because this assumption is not completely true, given that every indicator demonstrates loss over time, serial blood sampling is necessary to allow a corrective mathematical regression of values to time 0, the theoretical concentration of the indicator prior to any removal from the vascular space.[7,8,13,25,32]

The overall goal of the current study was to offer a practical means for estimating the BV of rhesus macaques that can be applied accurately over a range of body conditions. The first aim was to determine the most suitable method for measuring BV. We compared 2 indicators (that is, tracers)—^{125}I-labeled rhesus serum albumin (^{125}I-RhSA) and fluorescein isothiocyanate-labeled hydroxyethyl starch (FITC–HES)—for simultaneous BV determination in a study group of rhesus macaques exhibiting a range of body conditions. The second aim was to determine the effects of sex, age, and body condition on BV in rhesus macaques.

Materials and Methods

Animals. All animals were housed at the Oregon National Primate Research Center, an AAALAC-accredited facility. All animal procedures were conducted at this facility in accordance with the Public Health Services Policy on Humane Care and Use of Laboratory Animals and approved by the IACUC. This study used 20 (10 male, 10 female) healthy adult Indian-origin rhesus macaques (age: mean, 12.5 y; range, 5.7 to 17.6 y; weight: mean, 9.7 kg; range, 5.2 to 22.9 kg). All macaques were born in captivity: 17 were born at the Oregon National Primate Research Center; the remaining 3 were born at breeding facilities within the United States and later transferred to the research center. All animals were either single- (n = 8) or pair- (n = 12) housed in cages appropriate for their sizes. Fourteen of the macaques were considered to be SPF for simian retrovirus, SIV, simian T-cell leukemia virus, and *Cercopithecinae herpesvirus* 1; the other 6 were nonSPF. The study population was selected to include a range of ages and body conditions according to a review of the animal records and visual assessment. A physical examination and medical records review were completed by a veterinarian for each macaque prior to the study. All macaques were deemed healthy, with no evidence of preexisting disease. Hct values obtained during the study were within normal limits (33% to 44%) in all of the animal subjects. No blood was withdrawn from subjects in the 21 d prior to the study, and no blood samples greater than 10 mL were taken from any of the subjects within 90 d of the study. Each macaque received potassium iodide (32.5 mg PO daily; Thyrosafe, Recipharm, Honey Brook, PA) administered for 5 consecutive days beginning 3 d prior to the procedure, to prevent radioactive iodine uptake by the thyroid gland. Macaques were fasted for 12 h prior to anesthesia.

Preparation of FITC–HES. FITC–HES was produced in a similar manner to that described previously.[30] The following protocol was conducted by using sterilized equipment and aseptic technique under a designated laminar flow hood. Putrescine dihydrochloride (4.0 g) was reacted with 100 mL of 6% HES in 0.9% sodium chloride (Hespan, B Braun Medical, Irvine, CA) in the presence of borane pyridine complex (1.0 mL) at pH 7.0 for 14 d. The modified starch was precipitated with absolute ethanol (255 mL) and centrifuged at 1125 × g for 20 min (model TJ-6, Beckman Coulter Life Sciences, Indianapolis, IN), the supernatant removed via vacuum aspiration from the pellet, and the precipitate resuspended in 80 mL sterile water. Free putrescine and alcohol were removed by exhaustive dialysis against sterile water. The resulting complex was dissolved in 100 mL sterile water. The pH was adjusted to 9.0 by adding approximately 50 mL saturated aqueous disodium tetraborate and was mixed with FITC (300 mg) for 2 d. The fluorescent starch (FITC–HES) was then precipitated and washed in absolute ethanol before removing unreacted FITC by exhaustive dialysis against sterile water. Size-exclusion chromatography was used to confirm that all unbound FITC had been removed by dialysis. The elution volume of FITC–HES was equivalent to the void volume of a 25G Sephadex gel chromatography column (1 cm × 10 cm, void volume determined by blue dextran elution). The FITC–HES was then lyophilized in 26-mg aliquots, each of which was

reconstituted with sterile water immediately prior to dosing. A sample from each aliquot was submitted to the laboratory for serial dilution to determine the fluorescence concentration of the injected material and to serve as an in vitro standard for the in vivo experiment. Subjects received a 4-mL dose containing either 10 mg or 20 mg FITC–HES by intravenous injection.

Preparation of ^{125}I-RhSA. The radioiodination procedure was similar to that described previously.[28] Briefly, purified rhesus monkey serum albumin (RhSA, EquitechBio, Kerrville, TX) was diluted to 1 µg/µL with 0.05 M sodium phosphate buffer, pH 7.5, in aliquots of 20 µL and stored at –80 °C until radioiodination. Iodination was achieved by using the traditional chloramine T method with 20 µg RhSA, 1 mCi ^{125}I (MP Biomedicals, Santa Ana, CA), and 30 µg of chloramine T. The reaction was stopped after 1 min by adding 190 µg of sodium metabisulphite. Free ^{125}I was separated from bound ^{125}I-labeled RhSA by collecting 1-mL fractions from a column (1 cm × 10 cm) loaded with 10 mL of BioGel P60 (catalog no. 150-4160, BioRad, Hercules, CA). The peak of ^{125}I-RhSA appeared in fraction 4, whereas the free ^{125}I eluted after fraction 6. The estimated amount of ^{125}I-RhSA in fraction 4 based on the radioactivity and elution pattern was about 11 µg. Fraction 4 was separated into aliquots of 250 µL and stored at –20 C for use within 4 wk. Subjects received a 5-mL dose containing approximately 0.1 µg ^{125}I-RhSA (about 3×10^6 counts per min) in sterile saline.

Anesthesia, intravenous catheterization, and baseline sample collection. Each subject was sedated with an intramuscular injection of ketamine (5 to 10 mg/kg; Ketathesia, Butler Schein Animal Health, Dublin, OH), and then anesthesia was maintained with isoflurane (Piramal Healthcare Limited, Andhra, India) 1 to 2 Vol% in 100% oxygen administered through an endotracheal tube. Subjects were placed in dorsal recumbency, and 3 intravenous catheters were inserted: one in each cephalic vein and one in the left saphenous vein. The left saphenous catheter was used only for blood sample collection. A 3-mL blood sample was collected from the saphenous vein catheter for determination of Hct and baseline fluorescence. Hct was measured on an automated analyzer (Hemavet 950 FS, Drew Scientific, Waterbury, CT).

Body composition assessment. While anesthetized and just prior to administration of the tracer agents, each subject was weighed (in kg), measured (in cm, crown to rump), and assessed for body condition score (BCS) on a standardized scale.[4,29] Surface area and body mass index were calculated by using the following equations that relate body weight (BW) to crown-to-rump length (CRL):[19]

$$\text{surface area} = \left(\text{body weight}^{0.6046}\right)\left(\text{crown to rump length}^{0.1862}\right)(514) \;/\; 10{,}000$$

$$\text{BMI} = \text{body weight} \;/\; \left(\text{crown to rump length} \;/\; 100\right)^2$$

Next, body composition was determined using dual-energy X-ray absorptiometry (DXA) (Discovery A, Hologic, Bedford, MA). DXA analysis was performed twice for each subject and the 2 values for each measurement averaged.

Tracer administration and blood-sample collection. Doses of ^{125}I RhSA and FITC–HES were injected simultaneously into the 2 cephalic vein catheters, one tracer per vein, followed by 3 mL heparinized saline (0.9% NaCl). Blood samples were collected from the saphenous vein catheter at 12, 18, 24, 30, and 36 min after injection, by using a dual 3-way stopcock device to allow the removal of a 3-mL presample and then a 3-mL blood sample for analysis, followed by reinfusion of the presample and flushing with 3 mL lactated Ringer solution at each time point. Blood collection tubes (3-mL EDTA vacuum phlebotomy tubes, Becton Dickinson, Franklin Lakes, NJ) were kept in a dark container until all samples from the subject were collected. After collection of the final blood sample for analysis, anesthesia was discontinued, all intravenous catheters were removed, and each animal subject was allowed to recover. Blood tubes were then centrifuged at 1430 × *g* for 10 min (model GPKR 356517, Beckman Coulter Life Sciences) and the plasma removed by using an extended fine-tip, small-bulb, disposable pipette. The plasma concentrations of radioactivity and fluorescence were then determined as described.

Plasma volume measurement by using ^{125}I-RhSA. Before each study, the radioactivity of the ^{125}I-RhSA to be injected and the calibration of the gamma counter were confirmed by making a 12-step dilution (in saline) from 1 mL of the ^{125}I-RhSA (1:1, 1:50, 1;100, 1:200, 1:300, 1:400, 1:500, 1:600, 1:700, 1:800, 1:900, and 1:1000). The variation at each dilution between separate studies was less than 5%. For each study, 1.0-mL plasma samples at each time point were placed into 12×75-mm glass test tubes for assessment of ^{125}I activity (counts per min) by the gamma counter (model 5005, Cobra, Hewlett Packard, Downers Grove, IL). The concentration of ^{125}I-RhSA at time 0 was extrapolated by linear regression of the radioactivity from the plasma samples obtained at the 5 time points. Plasma volume (as estimated by the volume of distribution of ^{125}I-RhSA) was calculated as described later.

Plasma volume measurement using FITC–HES. Before each study, the fluorescence of the FITC–HES to be injected and calibration of the fluorometer (FilterMax F3, Molecular Devices, Sunnyvale, CA) were confirmed by making a serial dilution (1:50, 1:100, 1:200, 1:400, and 1:800) in pooled rhesus macaque plasma. All dilutions (including a plasma blank) and readings were performed in duplicate at excitation and emission wavelengths of 485 and 535 nm, respectively. After adjustment for the fluorescence of the plasma blank, the resulting dilution curve was linear. The relative fluorescence units of the injected FITC–HES were calculated by averaging the product of each dilution factor and its blank-adjusted average. The fluorescence of plasma samples collected from subjects was measured in triplicate at each time point by using 150-µL plasma aliquots in black 96-well plates with clear well bottoms (Greiner Bio-One, Monroe, NC). The plates were top read by using the laboratory fluorometer (FilterMax F3, Molecular Devices) at excitation and emission wavelengths of 485 and 535 nm, respectively. The background fluorescence of the plasma, measured from the preinjection sample, was subtracted from each reading. The fluorescence concentration at time 0 was extrapolated by linear regression of the natural logarithms of the fluorescence values derived from plasma samples obtained at the 5 time points. Natural logarithms were used because HES has biexponential clearance.[21,30] The antilog of the result was then used to calculate the plasma volume (as estimated by the volume of distribution of FITC–HES).

Calculation of BV. For both methods, BV was calculated from PV and Hct. The volume of distribution was assumed to be equal to PV.

$$V_2 = \frac{C_1 V_1}{C_2}$$

$$BV = \frac{PV}{(1 - HCT)}$$

where V_2 is the plasma volume, C_1 is the radioactivity of the injected ^{125}I-RhSA or the fluorescence of FITC–HES injected, V_1 is the volume of the injected ^{125}I-RhSA or FITC–HES, and C_2 is the radioactivity or fluorescence calculated from the regression of postinjection plasma samples to time 0.

Statistics. Correlation analysis followed by Altman–Bland analysis was used to evaluate the agreement between BV (mL/kg) obtained by using FITC–HES and ^{125}I-RhSA tracer techniques. In addition to the correlation analysis, Altman–Bland analysis was performed to determine whether systematic bias due to the scale variation was present. Wilcoxon rank-sum tests were used to compare the difference in BV between male and female macaques for each of the techniques. A nonparametric test was used instead of a parametric test because the sample size of the 'sex' subset was too small to evaluate the distributional assumption for a parametric test, such as a 2-sample *t* test. Multivariate regression analysis was performed to establish a BV prediction model using potential predictors such as age, percentage body fat, body weight, and BCS. SAS 9.4 (SAS Institute, Cary, NC) was used for the statistical data analysis, and Statistica (version 12, StatSoft, Tulsa, OK) was used for figures. *P* values less than 0.05 were considered statistically significant.

Results

Estimates of BV by using the ^{125}I-RhSA and FITC–HES methods. When BV (mL/kg) in rhesus macaques determined by using FITC–HES was compared with BV (mL/kg) determined by using ^{125}I-RhSA, the correlation r value was 0.9028 (Figure 2 A). The BV (mean ± 1 SD) obtained by using the ^{125}I-RhSA method was 68.75 ± 13.80 mL/kg, whereas that obtained with the FITC–HES method was 55.8 ± 16.10 mL/kg. The consistent bias toward a larger BV calculated by using the ^{125}I-RhSA method compared with the FITC–HES method is shown in Figure 2 B.

Influence of sex, age, and body condition on BV determination. The study population demographics for sex (10M; 10F), age (mean 12.5 y; range 5.7 to 17.6 y), and body condition (DXA mean body fat 25.0%; range 9.5 to 48.3%) were evaluated as potential predictors of BV. No significant difference in mean BV (mL/kg) between male and female macaques was observed (Figure 3). Specifically, Wilcoxon rank-sum test *P* values for ^{125}I-RhSA and FITC–HES were 0.9999 and 0.5760, respectively. In addition, BV and age showed no significant correlation. The Pearson correlation value for ^{125}I-RhSA compared with age was –0.18 ($P = 0.4428$), and that for FITC–HES compared with age was –0.32 ($P = 0.1674$; Figure 4 A and B).

Body condition was assessed objectively by measuring the percentages of body fat, bone, and lean by DXA. The fat percentage showed a strong negative correlation with BV, indicating that body fat percentage and body weight have opposite effects on BV. The slope of the correlation line indicates for each 1% increase in body fat, there is an approximately 1 mL/kg decrease in blood volume (Figure 5).

The estimated relationships between blood volume and body fat percentage are described by the following equations. When using ^{125}I–RhSA as a tracer,

$$BV_{^{125}\text{I-RhSA}} = 90.418 - 0.883 \times \text{fat\%}$$

or for each 1% increase in fat, BV decreases by 0.883 mL/kg.

When using FITC–HES as a tracer,

$$BV_{\text{FITC-HES}} = 79.814 - 0.981 \times \text{fat\%}$$

or for each 1% increase in fat, BV decreases by 0.981 mL/kg.

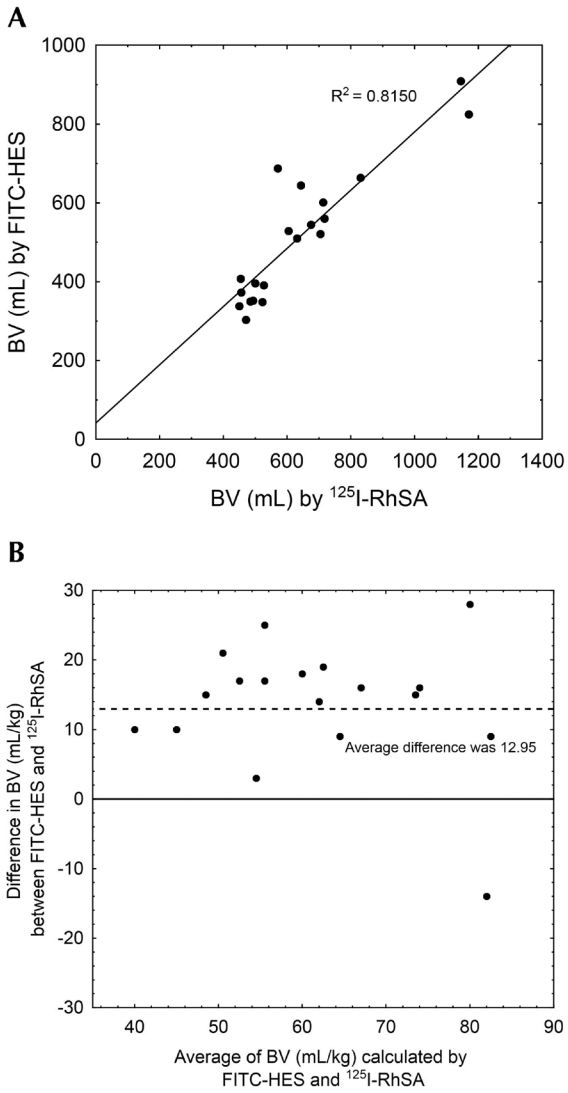

Figure 2. (A) Correlation between BV (mL) amounts obtained using the 2 methods: FITC–HES and ^{125}I-RhSA. (B) This Altman–Bland plot shows the difference relative to the average between the 2 methods of determining BV. The difference between the methods was consistent across the range of blood volumes in the study group. The average difference was 12.95 mL/kg, with bias toward a larger calculated BV with ^{125}I-RhSA method.

Evaluation of practical methods for body condition assessment. Because DXA may be an impractical method for determining body condition for routine BV calculation in large colonies, we evaluated other means of estimating body condition that might be used in the field more easily. SA was poorly correlated with DXA body fat percentage (Pearson r = 0.40) and BMI was only moderately correlated DXA body fat percentage (Pearson r = 0.63). However, the correlation of BCS with DXA body fat percentage was very good (Pearson r = 0.87, $P < 0.0001$; Figure 6), indicating that BCS can be used as a surrogate for DXA-determined fat percentage for predicting BV according to the following equation:

$$BV = 113.753 + (0.752 \times \text{body weight}) - (18.919 \times \text{BCS}).$$

When this equation was applied to the study group, the resulting BV and that measured by using FITC–HES showed reasonable correlation (Figure 7 A). The corresponding Altman–Bland plot indicates that all but one value fall within 2 SD

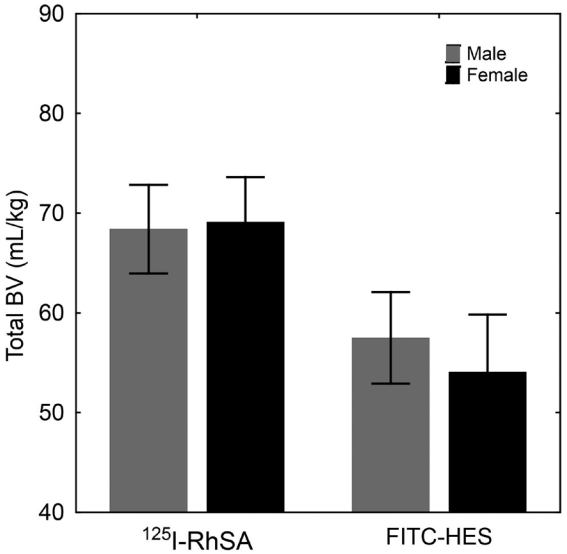

Figure 3. Mean BV (mL/kg) for male and female rhesus macaques using 2 methods, ^{125}I-RhSA and FITC–HES. There was no sex-associated difference in BV. Error bars, SEM.

of equivalency between measured and predicted values (Figure 7 B). Taken together, these results suggested BCS can be used as a practical surrogate method to estimate the percentage of body fat for the prediction of BV.

Discussion

In this study, we compared the volume of distribution of 2 different tracer substances (^{125}I–RhSA and FITC–HES) injected intravenously as a means of estimating PV. Hct and PV were then used to calculate BV. Because neither tracer is commercially available, we manufactured them in our laboratories as described in the Methods section. To our knowledge, this study is the first to describe the use of ^{125}I-RhSA or FITC–HES for determining BV in rhesus macaques.

When we compared the results from the 2 tracers, we found that the volume of distribution of ^{125}I–RhSA was greater than that of FITC–HES, so that using ^{125}I-RhSA resulted in greater BV values than did the FITC–HES tracer. This difference was consistent among all subjects, and no systematic bias emerged (Figure 2 B). Other investigators have observed this difference in volume of distribution between albumin and HES in human studies.[15,21,30] It is generally accepted that the measured volume of distribution of albumin exceeds PV.[15] This difference, which results in overestimation of PV (and therefore BV) when using a labeled albumin tracer, appears to reflect the albumin lost from plasma prior to 12 min (our first sample collection point) and not its measurable rate of loss over the ensuing 30 min. One plausible explanation is that albumin may bind to receptors on endothelial cells and therefore is removed immediately from circulating plasma.[15] In addition to the apparent bias of overestimation of PV when using the ^{125}I-RhSA tracer, other risks to the animal subjects should be considered. These risks include immunologic sensitization to the intravenous injection of heterologous rhesus protein and disease transmission associated with intravenous injection of a pooled blood product (rhesus serum albumin). Furthermore the subjects are exposed to doses (albeit very small) of radiation. For these reasons, we believe that the FITC–HES method provides a safer, more practical, and valid means of determining BV in rhesus macaques.

Another aim in our study was to evaluate factors that might affect blood volume as estimated by body weight, that is, sex,

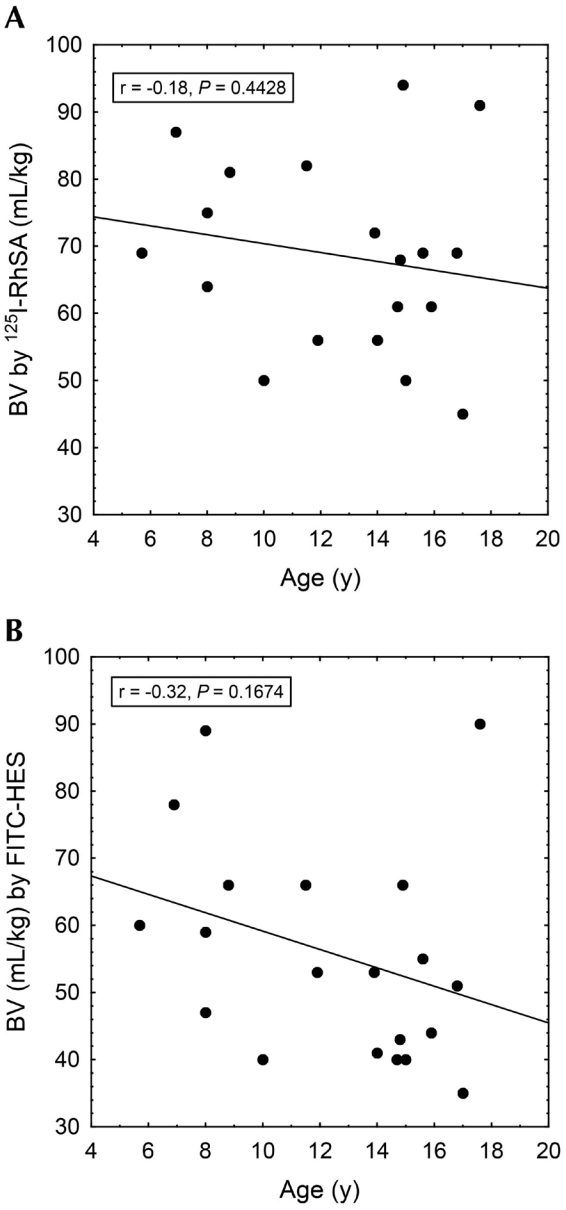

Figure 4. The influence of age (y) on BV (mL/kg) was assessed by using Pearson correlation for (A) ^{125}I-RhSA and (B) FITC–HES. Trends toward decreasing BV with increasing age were not statistically significant in the study group (age: mean, 12.5 y; range, 5.7 to 17.6 y).

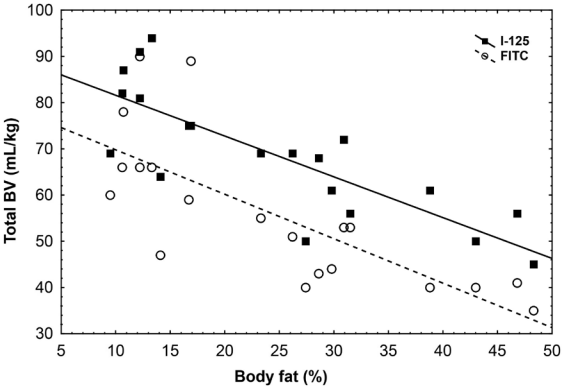

Figure 5. Correlation between BV and percentage body fat (Fat%), obtained via DXA. Total BV was measured by both the radioactive ^{125}I-RhSA method (squares) and the nonradioactive FITC–HES method (circles). For both methods, BV and percentage body fat were significantly negatively related ($P < 0.0001$ for both).

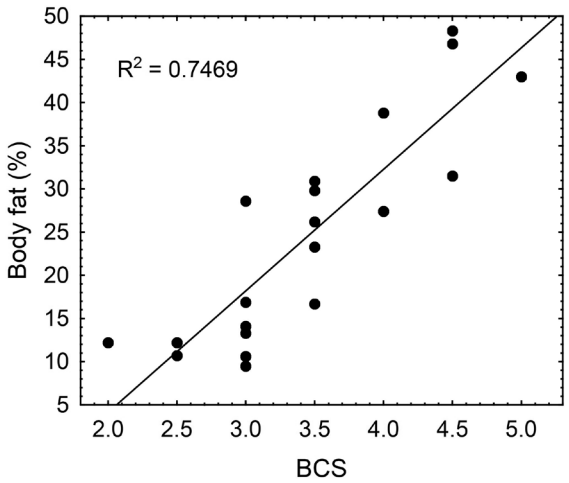

Figure 6. Average percentage body fat for each body condition score. The correlation indicates that the BCS can be used as a surrogate for body fat percentage in adult rhesus macaques.

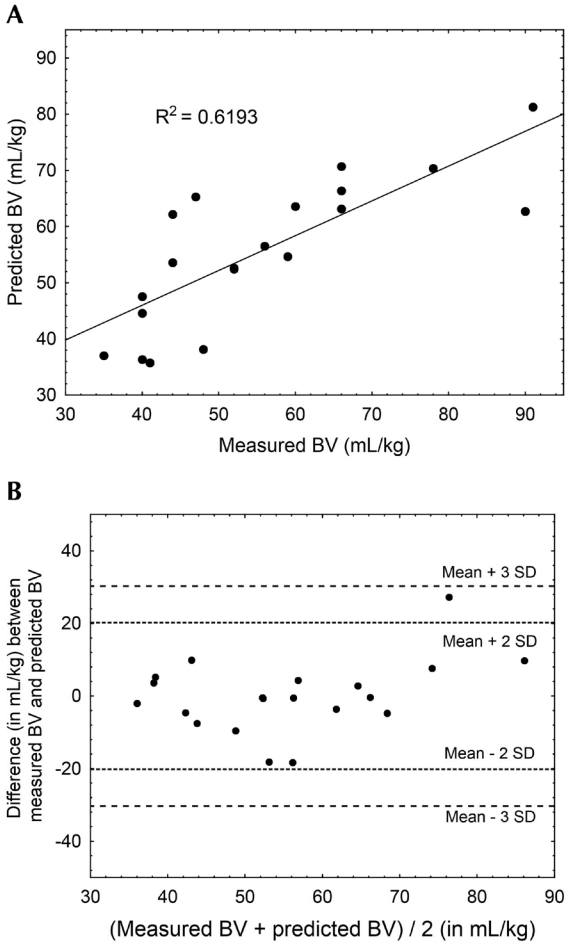

Figure 7. (A) Correlation between measured BV (FITC–HES method) and BV predicted by using equation 3. (B) The Altman–Bland plot shows reasonable agreement between the measured and predicted BV.

age, and body condition. Although sex has been determined to be a factor that significantly affects BV in humans,[8,18,27] we found no significant difference in the average BV (mL/kg) between male and female macaques in our study group (Figure 3). However, this finding may reflect the small sample size. Age is another factor believed to affect BV in humans.[5,16] Although we did not find a significant effect of age in our study group, a trend of decreasing BV with increasing age was evident (Figure 4). We suspect that this trend will become significant with the inclusion of subadult and geriatric animals in future studies. However, we found a strong correlation between body fat percentage and BV (Figure 5); this correlation is well-documented in the human medical literature.[9,18,23] Our models show that rhesus macaques categorized as having the 'optimum' BCS of 3.0 had approximately 18% body fat and 62.1 mL/kg BV (according to the FITC–HES method; 74.5 mL/kg by using the ^{125}I–RhSA method). Consequently, for each 1% increase in body fat, BV decreases by 0.962 mL/kg (or 0.883 mL/kg using ^{125}I-RhSA method). Unfortunately, body composition is not a factor considered in the fixed-ratio formulas currently used to estimate BV.

Body composition can be assessed objectively by using DXA analysis, which yields fat, lean, and bone percentages. However, DXA may be an impractical method of determining fat percentage for the purpose of BV calculation in large colonies. For this reason, we evaluated other, practical methods of estimating fat percentage or body composition. The method that correlated best with DXA fat percentage was the BCS system, a subjective semiquantitative method for approximating body fat and muscle proportions by palpation and visual assessment.[4] This system has been validated against DXA data in rhesus macaques.[29] The BCS scores correlated well with fat percentage in the current study (Figure 6), and the correlation was consistent with that published previously.[29] The BCS system is often used clinically to classify body condition, as a component of routine physical examinations. For these reasons, integrating the BCS score with body weight would be a relatively easy and prudent way to improve BV estimation for this species, and we have provided such a multivariate equation to this end. This equation represents BV with far more accuracy than any of the fixed ratios currently in use (Figure 1). Moreover, the equation can easily be integrated with current computing and data management technology in a way that minimizes user exposure to the background mathematics. The correlation between measured BV (FITC–HES method) and that predicted by using our BCS-containing equation (Figure 7) was reasonable (r^2 = 0.6193), given that we had only 20 subjects. We expect the correlation will improve with more subjects in future studies.

A particularly noteworthy finding was the profound overestimation of BV in obese animals (BCS > 4.0) when any of the widely used fixed-ratio formulas were applied. This finding may have important implications regarding animal wellbeing. For example, a 16-kg macaque with a BCS of 5.0 has a BV of 500 mL when calculated according to our BCS-containing equation, whereas the BV estimated by application of the 10%:10% rule is 1600 mL, a greater than 3-fold overestimation. This problem has become especially relevant with the recent interest in macaque models of obesity. In keeping with 3Rs principles,[26] the application of our proposed formula is an appropriate refinement to improve BV estimation when determining limitations to blood withdrawal, especially in protocols involving obese macaques.

In summary, the ultimate goal of this research is to better define the limits of benign blood withdrawal in rhesus macaques. These limits, intended to help prevent animal harm and experimental confounds, are likely best expressed as a proportion of an animal's total BV. In this study, we described a means of accurately measuring BV by using FITC–HES indicator-dilution methodology. We then identified significant predictors of BV, body weight and BCS, and proposed a formula that takes these factors into account to estimate BV more accurately. We hope

the findings of this study provide the first step from which investigators can evaluate the effects of various blood-collection proportions and frequencies. The results from similar future studies might be used to develop a unified evidence-based approach to defining BV collection standards among the institutions using rhesus macaques in biomedical research.

Acknowledgments

We thank Nikki Sternberger and Lindsay Bader for their outstanding animal handling and technical support. We truly appreciate the generosity and enthusiasm with which Jody Janovick shared her laboratory space and her expertise. We also thank Dr Monica Hinds for the use of her laboratory and facilities for handling radioactive substances as well as Dr Kristine Coleman for her greatly valued editorial advice.

This work was made possible by the Oregon National Primate Research Center core grant P51OD011092 from the NIH.

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