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Original Research

Variation in Organ Volumes of Matched BALB/c Mice by Microcomputed Tomography Analysis

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High-resolution microcomputed tomography technology has allowed researchers to use live mice to address questions that previously could be answered only at necropsy. Serial analyses of the same mouse allow tissue changes to be followed over time. The ability to follow a single mouse noninvasively can decrease the total number of mice required for the study. The magnitude of inter-mouse variation for matched mice undergoing microcomputed tomography has not been determined previously. We selected lung and contrast-enhanced stomach as tissues of standard size and anatomical structure that were hypothesized to vary minimally between mice. The analyses of the tissue volumes from matched mice showed considerable variation among mice, among multiple sequential scans of the same mouse, and even among multiple evaluations of the same scan. More variation occurred with repeated scans of the same mouse (intramouse variation) than between mice (intermouse variation). In addition, significant variation and obvious bias was detected between the 2 scan evaluators. These data suggest that to obtain the widest range of possible values, among which the true value would be found, multiple analyses of multiple scans of the same mouse must be performed by multiple scan evaluators.

Abbreviations: microCT, microcomputed tomography

High-resolution X-ray computed tomography (microCT) allows reduction of the number of animals used in studies of disease progression. This reduction is possible due to noninvasive longitudinal analysis, with the animal imaged, rather than euthanized for necropsy, at each selected time point. Because each animal is its own control, multiple analyses using microCT will reduce the number of animals required in a study and, as such, provide an opportunity for animal use reduction and refinement.[5]

Current applications of microCT include tracking bone repair and remodeling,[9] prostatic tumor growth,[12] and pulmonary changes associated with neoplasia and asthma.[3,7] In addition to these uses, microCT has been proposed as a rapid and sensitive tool for murine phenotyping, because mice can be scanned quickly and regions of anatomic variation identified through the use of imaging software.[4,12,14]

The output of a microCT scan is a series of 2-dimensional X-ray images that are reconstructed with computer imaging software to yield a 3-dimensional structure.[14] These images can then be evaluated using computer software to yield additional information about the structure. As with all X-ray images, the reliability with which a structure can be assessed is proportional to its visual contrast to the surrounding tissues. This contrast can be enhanced with high-density contrast media, such as barium or iodine.[11] Thus the resolution of a structure depends on multiple factors: the contrast of the structure with the surrounding tissue, the quality of the microCT image and image reconstruction, and the software analysis of the 3-dimensional structures.

To determine the necessary number of animals per study group, the variability of data measurements generated by a user must be established. Measurements can vary because of mouse to mouse anatomic differences (intermouse variation), equipment drift and instrumental variation, and subjective variability by the evaluator (intraevaluator variation). Once the reliability of the measurement is known, an estimate of sample variation can be determined, and the total number of animals needed in a study can be computed by use of power calculations.

To evaluate the question of reproducibility and repeatability of data generated, we determined the volumes of the stomach and lung through multiple analyses of multiple microCT scans for 10 mice. We selected the lung and stomach for evaluation in light of the inherent contrast present in the lung and the ability to easily add contrast to the stomach. The addition of contrast enhances the ability to differentiate voxels that belong to the organ of interest from those belonging to adjacent tissues. Serial determination of volumetric measurements of microCT images generated by using 4-wk-old female inbred BALB/c littermates provided the opportunity to assess the variation within a mouse, between mice, within an evaluator, and between evaluators. The data generated in this study may be applicable to other groups of genetically matched mice, although variation in organ sizes during times of rapid growth may affect the generalizability of this study.

Materials and Methods

Mice. Ten female 21-d-old littermate BALB/cAnNTac mice were purchased (Taconic, Germantown, NY) and allowed to acclimate for 7 d prior to study in a specific pathogen-free vivarium accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care, International. During the acclimation period, the mice were housed 5 per cage in sterile, individually ventilated, isolator cages (Alternative Design, Siloam Springs, AR) with hardwood chip bedding

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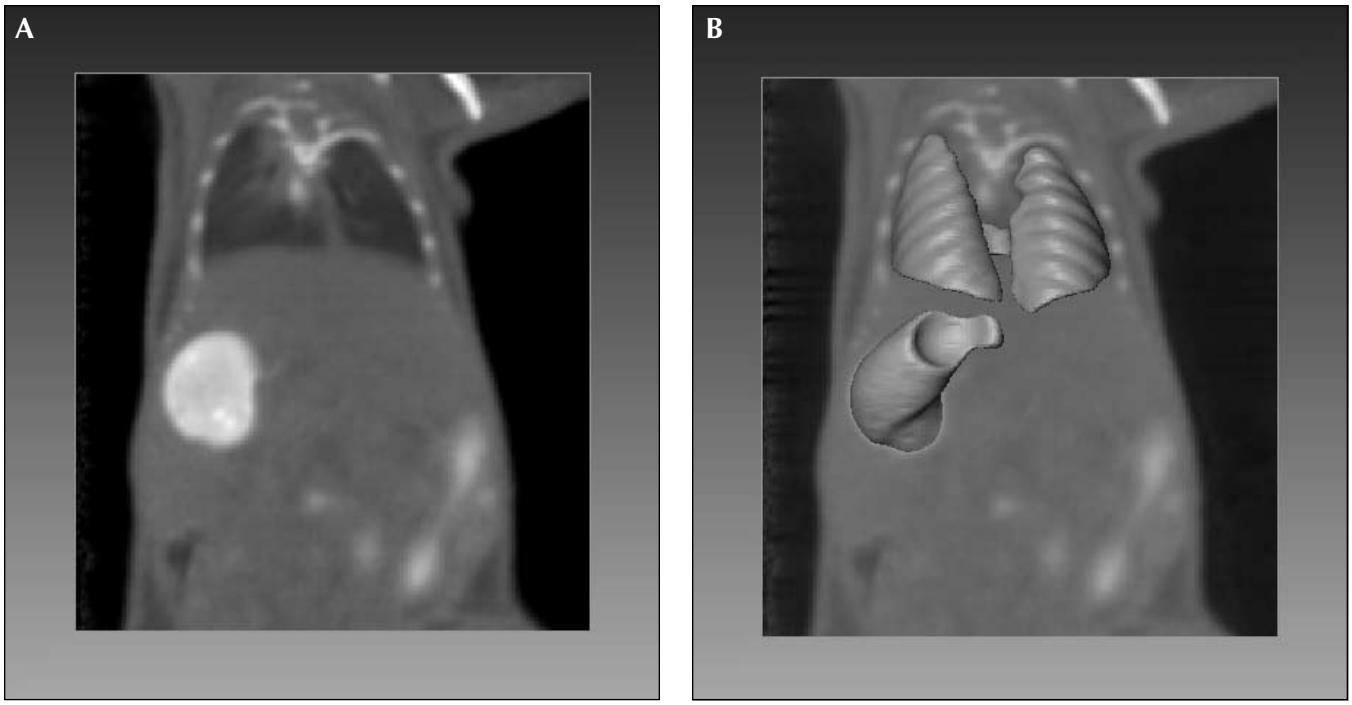

Figure 1. (A) Coronal view of mouse number 1 with iodinated contrast material in the stomach. (B) The same coronal view with 3-dimensional reconstruction of the lung and stomach.

(Northeastern Products Corp, Warrensburg, NY) and nesting material (Nestlet, Ancare, Bellmore, NY). Mice were provided with autoclaved pelleted rodent diet (NIH-31 Open Formula, Zeigler Bros, Gardners, PA) and acidified water. Mice were identified by tail tattoo to allow for randomization during image acquisition. The source facility was free of all known murine viruses, endoparasites, and ectoparasites. In addition, the following murine pathogenic bacteria were not found in the source facility: cilia-associated respiratory bacillus, *Citrobacter rodentium*, *Clostridium piliforme*, *Corynebacterium kutscheri*, *Helicobacter* sp., *Mycoplasma* sp., *Pasteurella pneumotropica*, *Salmonella* sp., and *Streptobacillus moniliformis*. All procedures were approved by the animal care and use committee in accordance with applicable federal regulations.

microCT image acquisition. The 10 littermate mice were divided into 2 groups of 5 mice each for the 2 scanning sessions. The 5 mice within each group were randomly assigned a scan order. Mice were not fasted prior to the study. At 5 min prior to anesthesia, mice were weighed and individually gavaged by use of a 22-gauge feeding needle with 0.3 ml (22 mg iodine) of an iodinated liquid contrast material (MD-GastroView, Mellinckrodt, St Louis, MO) that had been diluted 1:5 with sterile water. Mice were anesthetized with an intraperitoneal injection of 100 mg/kg ketamine (Ketaset, Fort Dodge, Fort Dodge, IA) combined with 10 mg/kg xylazine (AnaSed, Ben Venue Laboratories, Bedford, OH). Once anesthetized, mice were placed in ventral recumbency on the microCT platform with their front legs pulled cranially to minimize extraneous tissue between the source and the detector.

Mice were scanned by use of a microCT (ImTek MicroCAT II, Knoxville, TN) with the following parameters: 70 kVp, 480 µA, 175 µsec, with 360 projections with an isotropic voxel size of 0.128 mm without respiratory or cardiac gating. We acquired 3 sequential scans of each mouse, with each scan lasting approximately 7 min and the next scan begun immediately after the preceding scan ended. The 2-dimensional image slices were reconstructed with Feldkamp cone-beam reconstruction software (Imtek). Mice were weighed and euthanized after the scans were completed.

Irradiation dose. The dose of irradiation that was administered during this study was determined by placing a pen dosimeter (Bendix Aviation Corp, Cincinnati, OH) within the microCT scanner and repeating the scans with the same parameters.

Image analysis. Image slices were loaded into Amira 3.1 software (TGS, San Diego, CA), and voxel size was set to 0.128 mm. The images were cropped from the mandibular ramus to the caudal edge of the cecum, to focus on the thorax and cranial abdomen. By use of the Label Field function of the software, each image slice was assessed by means of a threshold-based function, which allowed assignment of voxels to a tissue of contiguous areas (that is, lung or stomach) within a range of voxel values. Manual contouring then was used to include areas of the stomach, identified by the evaluator, that were not incorporated by use of the threshold function. Assessment of all slices resulted in a segmentation set that when compiled, using the Surface Generation and Surface View functions of the software, produced a 3-dimensional volume reconstruction of the stomach and lung (Figure 1). The volume of the lung and the stomach for each analysis was determined with the Amira TissueStatistics function, which calculates the organ volume by multiplying the number of voxels in the labeled field by the size of a single voxel. All scans were analyzed independently in triplicate. The images from 3 randomly selected mice also were analyzed by a novel evaluator to determine inter-evaluator differences.

Histopathologic analysis. Mice were euthanized by cervical dislocation prior to recovery from anesthesia, and a comprehensive necropsy was performed. Cervical dislocation, rather than carbon dioxide asphyxiation, was used to minimize pulmonary changes that might confound histopathologic interpretation. The lungs were inflated with formalin in situ. The heart, lungs, and stomach were examined grossly and retained for histopathology to identify pathologic causes of volume differences between mice. Tissues were preserved in 10% neutral buffered formalin prior to dehydration in an alcohol series and paraffin embedding. Tissue sections were stained with hematoxylin and

Table 1. Mouse weight and organ mean volume and standard deviation (SDs) based on triplicate analyses of each scan of all mice (9 analyses/mouse)

Mouse no.		Stomach volume (µl)						Lung volume (µl)					
		Scan 1		Scan 2		Scan 3		Scan 1		Scan 2		Scan 3	
	Weight (g)	Mean	1 SD	Mean	1 SD	Mean	1 SD	Mean	1 SD	Mean	1 SD	Mean	1 SD
1	15.9	304	12	259	1	224	8	344	21	324	19	315	25
2	17.1	503	2	345	9	281	19	395	61	377	58	319	16
3	14.2	255	10	147	15	126	5	414	71	370	63	267	83
4	15.8	502	32	455	12	347	13	466	92	442	88	422	74
5	14.4	342	4	266	7	270	3	387	24	325	20	335	8
6	15.8	390	15	354	71	320	11	461	24	390	21	405	23
7	15.2	333	7	230	8	220	12	407	81	354	70	327	14
8	15.6	361	0	287	18	284	5	305	44	335	49	367	50
9	15.0	369	17	322	7	305	5	414	33	379	30	356	46
10	15.1	265	4	191	11	200	4	341	20	317	19	297	24
Average	15.4	362		286		308		393		361		341	

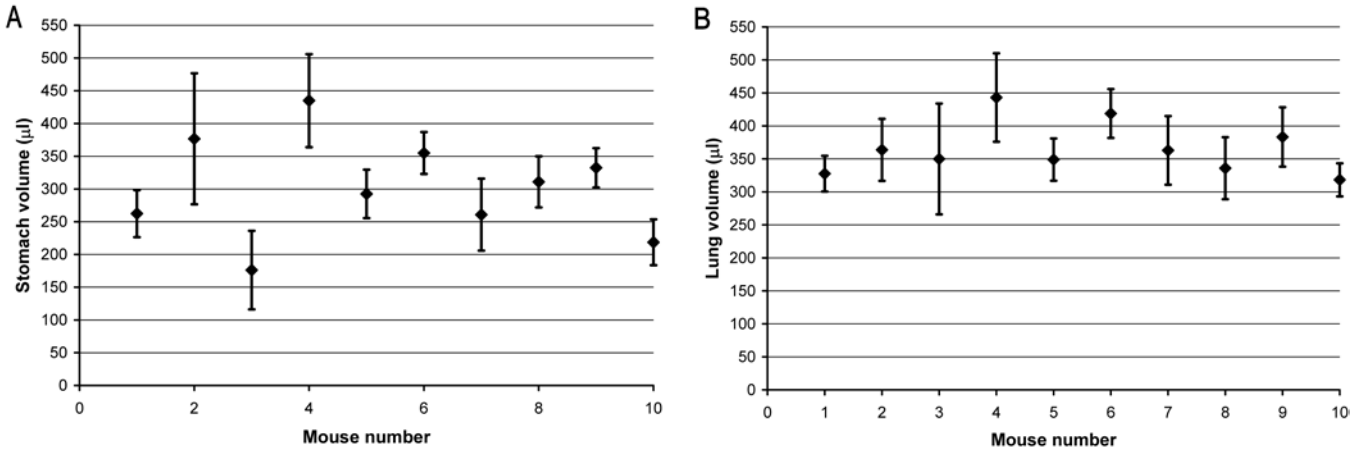

Figure 2. The mean volume (♦) was calculated from the 3 scans, read in triplicate, of each mouse for both the (A) stomach and (B) lung. The error bars reflect 1 standard deviation from the mean.

eosin and evaluated by a pathologist.

Statistical analysis. Using Microsoft Excel (Microsoft Office Professional Edition, 2003, Microsoft Corporation, Redmond, WA) we determined the arithmetic mean and standard deviation for the stomach and lung volume measurements of each mouse. All additional statistical analyses were based on a volume-per-gram basis to remove the potentially confounding variable of body weight. For discussion, the volume per gram was normalized to the typical weight of a mouse (20 g). The reproducibility coefficient of variation (standard deviation divided by the mean expressed as a percentage) was calculated by using Excel.

The remaining statistical analyses were conducted with the SAS programs (version 9.1.3, Cary, NC). Because the evaluator effect was categorical, with no correlation between the evaluators, the contribution of the volumetric measurements to the overall variance was calculated by using the NESTED procedure. The scan-timing effect was covariate with stomach volume, which decreased over time in a monotone fashion, requiring the MIXED procedure to assess differences in the volumes over repeated scans. The General Linear Model procedure, a generalized version of analysis of variance, was used to determine differences between scan evaluators. This model assumes that all experimentally applied variables can be controlled by the experimenters, such as time of scan postgavage of contrast material. These fixed variables are in contrast to the random variables, such as mice and evaluators, which are representatives of larger populations and cannot be reproduced exactly in future studies.

Results

Scans of the mice. An individual scan required approximately 7 min, with a total anesthesia time for the completion of 3 scans of approximately 28 min. The ketamine and xylazine doses given provided anesthesia for the entire scan, without the need for a supplemental dose. The mice received a radiation dose of less than 0.1 Gy.

Necropsy results. Gross necropsy of each mouse and histopathologic analysis of the lungs, heart, and stomach appeared normal and did not show evidence of space-occupying lesions that might influence the volumes calculated.

Image analysis. Adjacent areas of similar voxel valuation, equivalent to the same tissue density, were assigned to the same tissue by use of a threshold-based function. Voxel values are based on a continuous gray scale, with radiolucent air-filled regions having a smaller numerical value than regions of increasing radiodensity. The evaluator also performed manual contouring to remove areas that, despite having voxel values within the set range, were deemed as not being part of the organ of interest. Similarly the evaluator used manual contouring to add areas identified as being part of the organ. During determination of the lung volume, any radiolucent regions cranial to the pulmonary cupolas and caudal to the diaphragm were discarded. These radiolucent regions were from air in the trachea, in the stomach and esophagus from the gavage procedure, or from gasping during anesthesia induction. Air in the trachea caudal to the pulmonary cupolas was uniformly included in total lung volumes. Similarly esophageal and duodenal structures were

Table 2. Intramouse variability, expressed as the standard deviation (SD) and coefficient of variation (CV) per scan and per reading, of 10 mouse scans

	Stomach						Lung					
	Scan 1		Scan 2		Scan 3		Scan 1		Scan 2		Scan 3	
	SD (µl/g)	CV	SD (µl/g)	CV	SD (µl/g)	CV	SD (µl/g)	CV	SD (µl/g)	CV	SD (µl/g)	CV
1 reading per scan	4.71	20.12%	5.29	28.70%	4.05	24.31%	3.96	15.47%	3.59	15.31%	3.73	16.86%
3 readings per scan	4.65	19.88%	5.26	28.55%	4.02	24.13%	3.57	13.96%	2.45	10.42%	2.87	13.00%

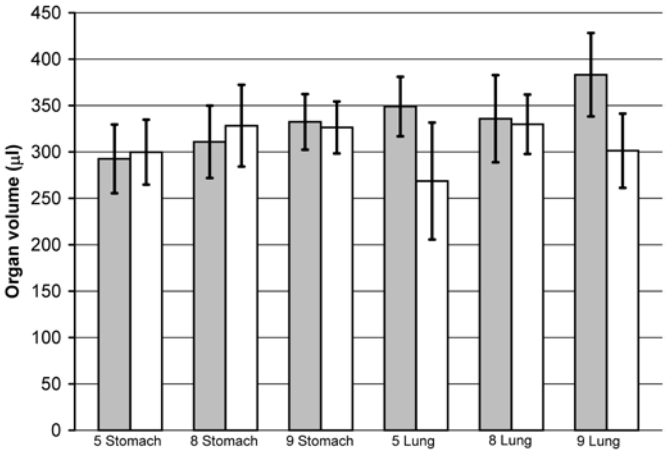

Figure 3. The lung volume for 3 randomly selected mice was determined by a triplicate analysis of each of 3 scans by 2 people. The novice evaluator (light bars) had a larger standard deviation range and lower total volume overall than did the experienced evaluator (dark bars). The error bars reflect 1 standard deviation from the mean.

not used in calculating total gastric volume. The gastrointestinal contrast agent coated the stomach walls, with progressive movement into the intestines over the sequential scans.

Mouse variability. The organ volume mean and standard deviation, obtained from the triplicate analyses performed by a single evaluator, is displayed for each scan of each mouse in Table 1. These calculations and the mean organ volume determined by evaluating all the scans for a single mouse (Figure 2) demonstrate that the triplicate analyses of the lung scans showed less variability among the different scans of the same mouse than among triplicate reads of the same scan. Analysis of the triplicate reads of the stomach scans showed comparable variation among the scans of all mice and within the scans of a single mouse. In addition, the standard deviation for organ volume was larger for the lung scans than for the stomach scans. Contributions to the overall lung volume variance using the NESTED procedure indicated that imprecision in the data was due as much to intramouse variation as to intermouse variation for the lungs, for which scan-to-scan variance was equal to that of mouse-to-mouse. This finding was in contrast to the variance for stomach volume, where less than 3% of the overall variability was due to read-to-read variance and the remainder due to mouse-to-mouse variance. Based on the data generated for overall variance by using all 10 mice (Table 2), the standard deviation for the average stomach volume was 4.65 µl/g mouse weight and that for lung was 2.53 µl/g. The coefficient of variation (Table 2) for the lung decreased by 20% and for the stomach by 1% when multiple scans were performed, with 3 analyses per scan. In contrast, the average stomach volume using a single read of each scan per evaluation provided a standard deviation of 4.71 µl/g mouse weight. The triplicate analysis of each scan yielded a standard deviation of 3.00 µl/g mouse weight for the lung.

Evaluator variability. When the volumes for the stomach and lung for 3 random mice were determined by 2 evaluators, greater variability and an obvious bias were seen for the lung but not the stomach (Figure 3). The effect of the evaluator was different for the analysis of the stomach and lung (Table 3). For the lung, interevaluator variance was greatest, whereas intermouse variability was negligible when compared with other sources of variation. For the stomach, a minor percentage of the variance was attributable to intra- and intermouse and interevaluator variability; most of the variability was attributed to intraevaluator variability. The general linear model procedure detected a statistically significant ($P = 0.0009$) average difference of 24% between the novice and experienced evaluator for the lung measurements but no bias between the evaluators on stomach measurements.

Organ volumes. The means and standard deviations for the lung and stomach volumes of each mouse are shown in Figure 2. The mean lung and stomach volumes for all 30 scans were 360 µl and 302 µl, respectively. When the volumes were calculated on a per gram basis and adjusted to a typical mouse body weight of 20 g, average lung and stomach volume increased to 475 µl and 390 µl, respectively. Further, 80% of the mice showed a noticeable decrease in stomach volume with sequential scans, necessitating use of the MIXED procedure for statistical analysis. For stomach measurements, a statistically significant ($P < 0.0001$) average decrease of 3.34 µl/g over a 7-min scan was calculated; for lung measurements, a statistically insignificant ($P > 0.05$) average decrease of 1.06 µl/g over a 7-min scan was seen.

Discussion

This study originally was designed to address the hypothesis that the amount of variation between microCT scans would be greater between mice and that repeated scans of the same mouse or repeated analysis of the same scan would have minimal or no variation when age-, sex-, and strain-matched mice were used. However, the data revealed that the majority of variation occurred when mice were repeatedly scanned and that this variation was related to differences in the scan analyses rather than to differences between mice. This variability occurred in the analyses of both stomach and lung volumes, but it was greater for lung. In addition, when multiple evaluators measured the lung volumes, an obvious bias was detected between the evaluators, and the lung volumes determined by each evaluator differed significantly.

Although this study was developed to examine the reproducibility of organ volume analysis, the lung and stomach volumes for the mice in this study were not confirmed using instrumentation such as a plethsymograph or helium dilution techniques[13,15] because of a lack of equipment and technical expertise. While published values for lungs exist,[8] those for the stomach volume of BALB/c mice are not available. Regardless, stomach volume varies not only with age but also with diet. Because the mice in this study were not fasted prior to being given oral liquid contrast media, the stomach volume determined likely was a function not only of time since food consumption and amount of food consumed but also a function of time and peristalsis since contrast was administered. The intramouse stomach volumes

Table 3. Organ variability, expressed as standard deviation (SD) and coefficient of variation (CV), based on scans of 3 mice by 2 evaluators

Type of variation	Stomach						Lung					
	Scan 1		Scan 2		Scan 3		Scan 1		Scan 2		Scan 3	
	SD (µl/g)	CV	SD (µl/g)	CV	SD (µl/g)	CV	SD (µl/g)	CV	SD (µl/g)	CV (µl/g)	SD (µl/g)	CV
Intermouse	0.00	0.00%	0.90	4.44%	1.30	6.76%	0.00	0.00%	0.00	0.00%	0.00	0.00%
Intraevaluator	1.25	5.30%	1.54	7.62%	0.94	4.87%	3.11	13.33%	1.97	9.19%	2.27	10.91%
Interevaluator	0.80	3.38%	0.80	3.97%	0.00	0.00%	3.00	12.86%	2.66	12.44%	4.02	19.29%
Overall	1.48	6.29%	1.95	9.68%	1.60	8.33%	4.32	18.52%	3.31	15.46%	4.62	22.16%

might differ if the scans were performed on sequential days or at markedly different times of the same day, reflecting differing amounts of ingesta in the stomach. Although the volume of contrast agent given was close to the total calculated stomach volume, the maximal stomach volume must be greater still, because overflow of the contrast material back into the esophagus was not noted.

The variability in the stomach volume was time-dependent, perhaps reflecting the gastrointestinal motility moving contrast agent from the stomach into the intestinal tract. This trend toward time dependency was apparent in the scan analyses; as time since anesthesia induction increased, more contrast was seen in the duodenum. Lung volumes did not show time dependency, suggesting a constant respiratory rate that was not affected by the time since induction of anesthesia.

In this study, the majority of the variation observed for the lung was intramouse rather than intermouse. Intramouse variation of the lung can be reduced by performing supplementary scans of the same mouse. Other factors resulting in intermouse variability may be the anatomic dissimilarity of the age-, sex-, and strain-matched mice or perhaps the inherent variation that arose from independent analyses. This variation can be addressed by developing standards and rigorous training to minimize the variation seen or by obtaining the widest range of possible values among which the true value is found. Either of these methods allows detection of significant differences between groups of mice. The disadvantage of the latter method is that a greater difference between groups, possibly requiring assessment of more mice, must be found to overcome the variability seen.

Many methods can be used to overcome variability in organ volumes, including the use of iodinated contrast.[6] Xenon gas can be used in the pulmonary system to increase the contrast between air-filled spaces and soft tissue vasculature.[15] Pulmonary gating with microCT has been shown to decrease the variability in organ volumes when threshold-based analysis is used.[6] Gating with the microCT coordinates the acquisition of the image with the respiratory or cardiac cycle; for example all images would be gathered during the middle of the expiratory cycle. Although gating decreases the movement artifact associated with breathing, it requires expensive equipment and increases the scan acquisition time fourfold. This increase in time would limit the total number of animals that could be scanned on a given day.

Repeated scans and analysis of each scan increase the need for computer storage space and analysis time. Lack of computer storage space and time for analysis might become a limitation because each scan requires more than 100 megabytes and an average of 45 min of analysis.[1] In addition, multiple readers for each scan may be necessary to minimize bias from a single reader and to improve the precision of the measurement, especially for the lung. Another disadvantage of repeat scanning of a single mouse is repeated doses of X-ray beam radiation. Excessive doses of radiation might affect the health of the mouse and the experimental outcome.[2,3,4] The mice in the present study all received far less than the 50% lethal dose of 7.85 Gy.[10]

Our study shows that microCT can continue to be viewed as a method for both refinement and reduction of animal usage and, through minimization of the intermouse variation, by performing multiple scans and analysis of the scans, that the number of mice per study can be reduced. Determining the reproducibility of volume measurements is an initial step in calculating the number of animals needed in a study.

The number of animals required for a study, n, can be determined using a power calculation: $n = \kappa [(z_{1-\alpha} + z_{1-\beta})(\sigma/\delta)]$.[2] This formula is for a single treatment group, for which half of the animals are in the control group and the other half are in the treatment group and the mouse serves as its own control (κ = 1). The ability of the mouse to serve as its own control is a major reduction and allows the number of mice (n) to be reduced by 2. The α value, the false-positive risk level, is typically set at 5%, and the β value, false-negative risk level, is typically set at 10%. The σ value, standard deviation, in light of the overall variance calculated in the current study, is 4.7 µl/g for the stomach and 3 µl/g for the lung. The δ value is a cut-off below which the difference is considered by the experimenter to be statistically insignificant. According to the formula given and with assumption of a 1-tailed test and a δ of 3 µl/g for the stomach volume, a study evaluating the effect of a single treatment on the stomach volume would require a total of 21 mice, with each mouse serving as its own control.

Our study has shown the variability inherent in the assessment of the stomach and lung of young BALB/c female mice by use of microCT analysis. These data are crucial for determining the number of mice to be used in any subsequent studies and suggest that although microCT affords refinement and reduction, pilot studies to determine the variability in the assessment parameters are essential.

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