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Investigation of Various Intramuscular Volumes Delivered to the Semimembranosus Muscle of *Cavia porcellus*

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The goal of this study is to provide quantitative data on the ideal volume for intramuscular (IM) injections into the semimembranosus muscle of guinea pigs weighing between 320 to 410 grams. This evaluation comprised 2 experiments. The first was to assess dispersion leakage of intramuscularly injected iohexol, a radiocontrast agent commonly used in Computed Tomography (CT), based on analysis of in vivo imaging. The second used varying volumes of intramuscularly injected sodium chloride (0.9% NaCl) to assess pain and pathology associated with IM injection. Hartley guinea pigs were injected IM with varying volumes of either iohexol or sodium chloride (150, 300, 500, 1000 and 1500 µL). In the iohexol experiment, results suggest IM volumes of 150 and 300 µL remain within the target muscle. In the experiment using sodium chloride, pain and pathology did not increase as IM volume increased. The pathology noted was related to needle tract through the musculature rather than the volume size of the injectate. The results did not reveal a correlation between volume of IM 0.9% NaCl and pain levels. We conclude that volume size correlates more with precision and accuracy of delivery into the intended muscle tissue. Regarding tissue distribution, our findings also suggest that the optimal capacity for IM injection in the semimembranosus muscle should be less than 500 µL.

Abbreviations: IM, intramuscular; CT, computed tomography

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Guinea pigs are one of the USDA covered species most frequently used in research, with much of their popularity attributed to their docile temperament, commercial availability, and easy maintenance.[5,31] They are also one of the most commonly used rodents in vaccine research.[4] A majority of these vaccines are administered through 2 major routes; subcutaneously and intramuscularly. When evaluating volume, one must consider multiple effects on the tissue being injected. These effects include pain, inflammation, damage to the muscle and optimal absorption without excessive dispersion within or outside of the intended target tissue. Recommended intramuscular injection volumes for the guinea pig range from 100 to 1000 µL at one single muscle site.[6,9,12,27,28] Many of these recommendations did not account for the varying size, age or weight of the guinea pig. In this experiment, we used 4 to 10 wk old guinea pigs that were 320 to 410 g in size. This study sought to determine the optimal volume for intramuscular injections, with respect to injectate dispersion, pain and inflammation in guinea pigs.

Refinement is one of the 3 major guiding principles for the ethical use of animals in biomedical research. The goal is to minimize pain and distress of the animal in research.[11] Efficiency and effectiveness of even a simple intramuscular injection can vary in every species. In a recent study, in vivo CT imaging was used to provide an in depth analysis regarding the evaluation of intramuscular (IM) injections in mice (*Mus musculus)*.[10] Using in vivo CT, they were able to dynamically assess the temporal biodistribution of iohexol injected into muscle. The major goal for this study was to use the same technology and equipment to evaluate current recommended intramuscular injection volumes in guinea pigs. Prior guidelines for IM injections in mice ranged from 1.5 µL up to 100 µL.[9-12,27,28] Recent research using CT in mice concluded that best practices for effective IM volume absorption was equal to or greater than 50 µL.[10] In that study, additional volume resulted in the dispersion of additional test material outside the intended muscle into the surrounding fascia and subcutaneous space.

Anatomic differences between species and size ranges of mammals may lead us to over- or under-dose animals which may have profound affect on the data derived from animal research. A great example of volume affecting research results was shown in a study using a high dose of intramuscularly injected botulinum-toxin A.[18] The aim of that particular study was to investigate what effect high doses of the toxin would have on the muscle and surrounding tissue. The authors found that the increased dose caused microstructural damage to the muscle tissue. This becomes particularly important when assessing the dispersion of an injectate to unintentional sites. Studies in rats have revealed that the rate of absorption after IM injection of a given substance is greatly affected by the volume of the solution, with both very small and large volumes eliciting a delay in absorption.[27]

Intramuscular injections are used frequently in biomedical research to deliver anesthetics, analgesics, antibiotics, and

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infectious agents. Research animals are also commonly used for the assessment of test articles intended for IM use in humans. One example of this is the strain 13 guinea pigs which were used for testing a DNA vaccine against both Lassa and Ebola virus for the development of protective immunity.[3] The rate and accuracy of administration of an IM injection could lead to inaccurate or distorted findings in such a study. Furthermore, any imprecision could complicate the interpretation of results in how the therapeutic levels would function in a human patient. It is critical to further assess the range of volumes that may be administered IM to common laboratory species and how these volumes impact the precision of delivery and systemic absorption of injected solutions.

Assessing pain in guinea pigs can be extremely difficult. In human medicine, conservation withdrawal is defined as a reaction to tangible or emotional antagonists in which the individual is likely to remove themselves from their environment.[17] Essentially, this is a way of preserving energy and collecting lost physical and mental stamina encountered during depression. Like most rodents, guinea pigs exhibit a 'conservation withdrawal' response and tend to hide overt signs of illness in the presence of an observer. This prey-species behavior makes the discernment of painful behaviors more difficult due to guinea pigs' natural tonic immobility.[8,13,31] Thus, clinical signs of pain may go unnoticed until the development of changes secondary to loss of normal behavior, such as dehydration, loss of body weight or body condition due to decreased consumptive behaviors, or unkempt hair coat due to decreased grooming behaviors. These signs of pain are nonspecific, may fail to indicate pain intensity, and are retrospective in nature.[13] An ethogram was created (Figure 1) by the study team, based on previous research regarding pain in guinea pigs, with the added observation of watching for signs of biting or licking (or both) at the injection site.[4,8]

The histopathologic changes seen after the intramuscular administration of saline have not been well documented.[29] Inflammation or damage to the local site can potentially be assessed through histopathology. Prior studies using iohexol injections in mice could not account for pathologic changes due to euthanizing mice 3 h after injection.[10] Recommended times for pathologic evaluation can range between 8 and 72 h ,and regeneration of muscle tissue is normally not seen before day 10.[29] Based on recommendations, the minimum time chosen to assess any type of pathologic change was 48 to 72 h.[29] In the current study, the euthanasia of guinea pigs that received iohexol prior to allowing time for an effective pathologic response was due to the potential acute renal toxicity caused by the iohexol itself.[19] Using allometric scaling, we compared the recommended pediatric safe dose of iohexol for a 3 mo old human child (intrathecal injection maximum 2 to 4 mL of 180 mg/iodine for a 6 kg child) to our guinea pigs.[7,23] We determined the maximum dose to be 132 mg of iohexol for guinea pigs weighing between 325 and 450 g. The dose was too low and the dilution would be too high to accurately compare a 150 µL dose to a 1500 µL dose. The decision was made to not dilute the iohexol solution (240 mg/mL). Iohexol is not designed to be given IM, but in a previous study, iohexol was effective in assessing dispersion of iodine in an injected site.[10] Due to potential postexposure effects of iohexol described in a prior experiment with mice,[10] and the potential pathologic effects of this solution on the muscle tissue, our experiment with guinea pigs included both iohexol IM injections with CT evaluation and a separate evaluation using sodium chloride IM injections.

Prior research has shown that injecting saline is an effective approach for examining pain and inflammation solely due to volume and needle variances. Saline was effective in focusing on volume related changes in pain and distress in mice, rats and rabbits.[29] Using anything other than a neutral medium like saline could add additional confounding variables to the result. As previously described, time after injection and the choice of medium is crucial to assess postinflammatory pathology.[29] Iohexol solution can cause necrosis; therefore using iohexol to address pathologic differences due to volume alone would not be informative.[29]

In this study, we administered IM injections into the semimembranosus muscles of 2 sets (n = 25 and n = 26) of guinea pigs. The first part of the study mirrored the original mouse study, but we injected both legs, rather than only one, with iohexol to assess dispersion by volume to 26 guinea pigs using CT (Figure 2).[10] Data from the first set of 26 guinea pigs would determine which volumes could be reliably delivered to the muscle without inadvertent delivery into the vasculature or leakage into adjacent tissue compartments. In the second part of the study, 25 guinea pigs were administered sodium chloride IM to the semimembranosus muscle to assess and compare both pain and inflammation responses specific to variations in volume. The major difference in the second experiment is we did not perform CT and only one leg received an IM injection as the contralateral leg served as the control. Both rear legs were submitted for histopathology to be assessed by a veterinary pathologist. These animals were monitored for 72 h after the sodium chloride injection using an ethogram to assess them for distress (Figure 1). In both portions of the study, the iohexol portion and the sodium chloride study guinea pigs were injected IM with a range of volumes that might be encountered in a research setting (150, 300, 500, 1000 and 1500 µL).

Using CT imaging during the first iteration of this experiment, we dynamically assessed the temporal biodistribution of the delivered volumes of iohexol in muscle. We hypothesized that as the injection volume increased, so would the risk of distribution of the injectate beyond the intended target tissue and of delayed systemic absorption from the muscle. The second part of the study (sodium chloride IM injection) we hypothesized that an increasing injection volume would be associated with greater muscular damage and inflammation in the target tissue. In addition, we hypothesized that increased volume would be associated with greater pain and decreased activity. The results of this study will support or allow refinement of the current guidelines for IM injection volume for this commonly used species.

Materials and Methods

Animals. Hartley guinea pigs (age, 4 to 10 wk; weight, 320 to 410 g; 27 male and 24 female) were obtained from Charles River Laboratories (Frederick, MD). All procedures were approved by the United States Army Medical Research Institute of Infectious Diseases IACUC.

Housing parameters were in accordance with the *Guide for the Care and use of Laboratory Animals*,[11] with all animals being housed in an AAALAC-accredited facility. All guinea pigs were socially housed in same-sex groupings of 2 to 3 animals in 20 × 20 × 8.5 inch stainless steel cages (Allentown Caging, Allentown, NJ; Lab Products, Seaford, DE), which were an open caging system. Guinea pigs were fed a fixed formula pelleted diet (2040 Teklad Global guinea pig diet 18% Protein, 33% carbohydrate, Envigo, Frederick, MD) and municipal water (on automatic watering systems with no further treatment) was provided without restriction by using an automatic watering system. Food enrichment items such as leafy greens, fruits, forage mix and

Parameter	Description	Score
Appearance	Normal smooth coat	0
	Ruffled Fur <25% of body (Excluding head)	1
	Ruffled Fur 25% to 50% of body	2
	Ruffled Fur >50% of body	3
Eye Opening	100 % open	0
	25% Closed	1
	50% Closed	2
	75% Closed	3
Breathing	Normal	0
	Rapid Breeding, no abdominal involvement	1
	Rapid Abdominal breathing	2
	Rapid abdominal breathing with pronounced effort	3
In-Cage activity	Animal moves around when cage is disturbed	0
	Animal moves around a bit, but quickly settles down	1
	Animal barely moves from its position	2
Mucous membrane color	Normal pink	0
	Pale white	1
	Blue or Purple, cyanotic	2
Activity during handling	Animal struggle to escape	0
	Struggles at first but quickly quiets	1
	Doesn't move in hand	2
Body Weight loss *Compare with initial weight	0% to 5% loss	0
	5% to 10% loss	1
	10% to 15% loss	2
	15% to 20% loss	3
Score	**Action**	
0-5	A minimum of twice daily observations	
8≥	Increase monitoring to three times daily*	
≥10	Moribund – Euthanize	

Figure 1. Guinea pig ethogram form for daily observations taken twice a day for 72 h (Actions were taken if there is any evidence of biting/licking at the site of injection. If the activity was noted it was placed on the daily observation sheets)

vegetables were provided twice a week along with enrichment devices to include certified guinea pig huts (Guinea Pig Hut Bio Serv, Product K3261). The cage bedding was cellulose (7070 C Teklad Certified Diamond Dry Cellulose Bedding, Envigo, Indianapolis, IN). The room temperature range was maintained at 68 to 79 °F (20.0 to 26.1 °C) with a set point of 74.5 °F (23.6 °C), and relative humidity was kept between 30% to 70% on a 12:12-h light:dark cycle.

Serum samples from dirty-bedding sentinel guinea pigs were tested quarterly (VRL, Rockville MD) for Sendai virus, Pneumonia virus of mice, Reovirus and Lymphocytic choriomeningitis virus. Additional quarterly testing included gross necropsy exams, PCR analysis of feces for *Helicobacter spp*., perianal tape testing for endoparasites, and fur pluck examinations for ectoparasites. Additional serology testing was performed annually to detect mouse adenovirus types 1 and 2, simian virus 5 and Reovirus type 1, 2 and 3. A complete necropsy with histopathologic examination by a veterinary pathologist was also performed yearly. All testing was negative throughout this study.

Drugs and materials. The first experiment used Iohexol. Iohexol (240mg/mL, Omnipaque 240, GE Healthcare, Chicago, IL) is an iodine-based radiopaque dye, commonly used in radiography and CT imaging studies and is labeled for intravenous use. We chose this radiocontrast agent for off-label intramuscular use because it is sufficiently concentrated to produce reliable imaging results at low volumes (150 µL) and due to various characteristics (pH, 6.8 to 7.7; osmolality of 520 mOsm/kg H_2O), it is less likely to cause irritation than other contrast agents.[1] Despite its lower irritancy, Iohexol has been known to cause potential muscular damage which is why it was not used in the second experiment. The second experiment used bacteriostatic

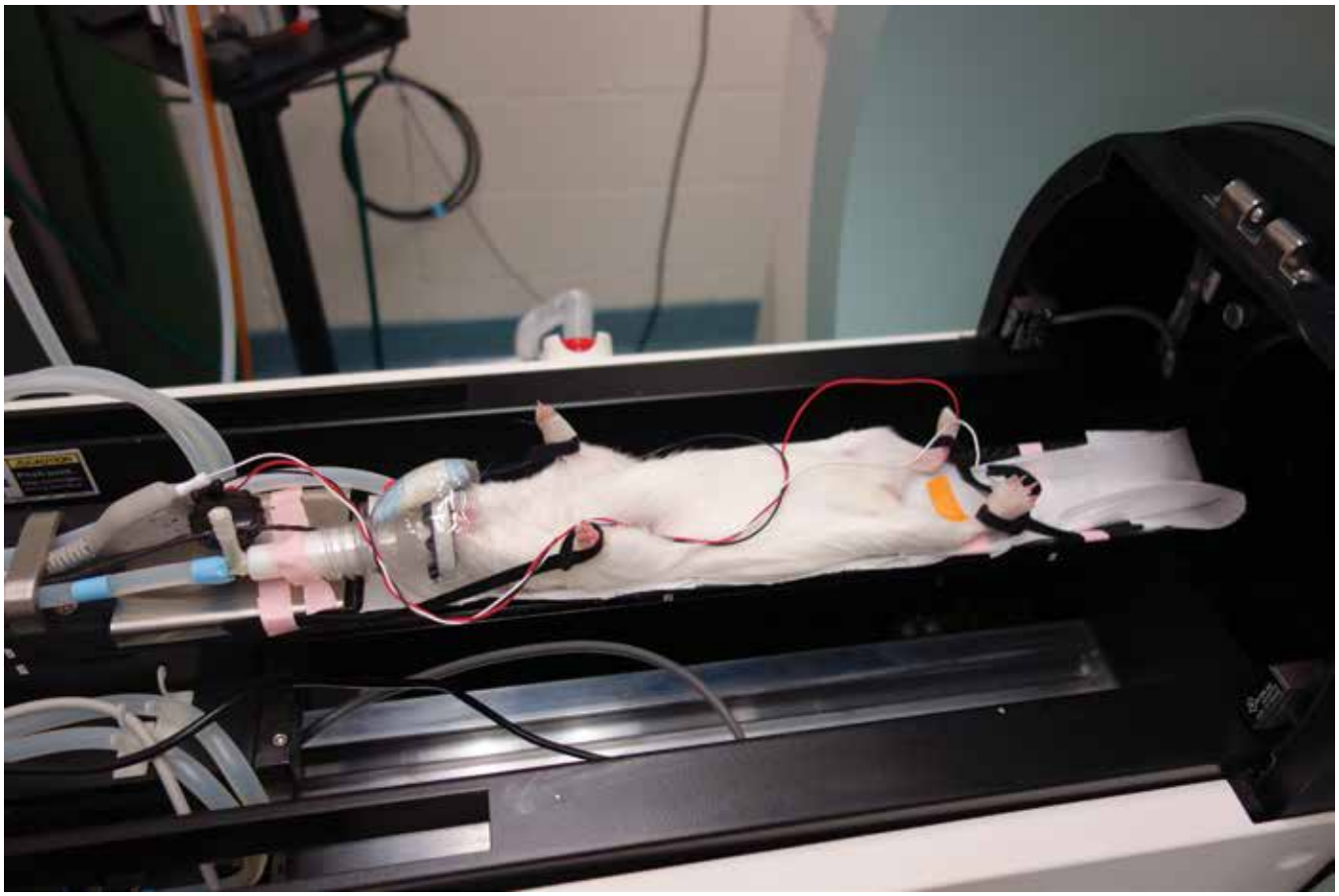

Figure 2. Guinea pigs were positioned for imaging in sternal recumbency. Anesthesia was maintained with a modified nose cone that scavenged waste anesthetic gas. The animal was secured for imaging with hook and eye fabric fasteners to ensure it remained still for imaging. The same 2 individuals positioned all guinea pigs throughout the entire study.

0.9% Sodium Chloride (30 mL Multiple-dose, Hospira, Lake Forest, IL). This normal saline reagent was isotonic to ensure any tissue damage observed within the muscle tissue would be related to volume rather than the substance being injected.[29]

For both experiments, we used Covidien Monoject needles and BD Luer-Lok syringes, both of which arecommonly used within veterinary research and practice. The materials used were the same for both the first and second experiment. For volumes less than or equal to 1000 µL, a 1 mL syringe BD Luer-Lok Tip was used. Volumes greater than 1000 µL used the Covidien Monoject 3 mL syringes (REF 309628). The same needle type BD Precision Glide 25 g × 5/8(0.5mm × 16mm) (REF 309628) was used regardless of volume size injected.

Guinea pigs used in the second experiment (sodium chloride) were implanted with IPT-300 antimigration Implantable Programmable Transponders (Bio Medic Data Systems chip size 14mm × 2mm) subcutaneously between the scapula, as per manufacturer recommendations, 3 d prior to beginning the experiment. These animals were coded and randomized by the biostatistician. The microchip was used to ensure the correct animal was evaluated during the 72 h observation period. The microchip was not used for the CT evaluation because of the potential visual distortion effects it could create on the CT images.

Intramuscular injection. A total of 26 guinea pigs (12 female and 14 male) were randomized by volume/sex/leg to receive one of the 5 assigned injection amounts (150, 300, 500, 1000 and 1500 µL) into each right and left hind leg. Broken down by volume,5 female and 5 males received 150 µL, 7 female and 8 males received 300 µL, 5 female and 6 males received 500 µL, 5 female and 6 males received 1000 µL, and 2 females and 3 males received 1500 µL. Placing a different amount of animals in each group was a statistical decision. The volume assignments were selected with 2 objectives in mind. The first was to maintain the total volume (sum of left and right legs) below levels which would be entirely too large. This prevents cases where a guinea pig gets 1000 µL in one leg and 1500 µL in the other. The second is that there is greater statistical power where 2 volumes are given to the same guinea pig. It is desirable to have the greatest statistical power when comparing volumes which are close together, and in the critical range of doses around 500 µL, which is approximately the current best practice volume. The net result is the design used in the previous mouse experiment.[9] The guinea pigs were anesthetized with Isoflurane (3% induction, 1% to 3% maintenance, Isoflurane, USP, IsoFlo Abbott Laboratories), by using an anesthetic vaporizer outfitted for guinea pig use (Integrated Multi-Patient Anesthetic Center [IMPAC6], VetEquip, Piney River, VA). Once anesthetized, the guinea pigs were placed in sternal recumbency on a tabletop, and checked for anesthetic depth by a negative toe pinch. All guinea pigs received an IM injection of Iohexol (240mg/mL) in the caudal thigh muscle (location of the semimembranosus muscle). The caudal thigh muscle was visually and manually isolated. The syringe plunger was aspirated to check for inadvertent placement within a blood vessel. Once the proper location was verified, the injection was delivered. Volumes assigned to each leg were coded and randomized by the assigned biostatistician. Each guinea pig received their assigned volume in the right and left hind legs. To prevent deviations in the

injection procedure, a single, proficient veterinary technician performed all of the intramuscular injections.

In the experiment using sodium chloride, 25 guinea pigs were removed from their cages and weighed individually. Guinea pigs were anesthetized and received IM injections as described above. They each received a microchip as described previously, 3 d prior to being given their assigned IM injection volume of sodium chloride. Microchipping occurred at the location of the dorsal scapula region in accordance with manufacturers recommendations (Bio Medic Data Systems). Volumes assigned to each leg were coded and randomized by the assigned biostatistician. The other hind leg was untouched, acting as a control.

CT imaging. Each guinea pig was injected intramuscularly with its assigned volume of iohexol into each hind leg. Guinea pigs were then positioned into the CT imaging trough (Figure 2) and maintained on isoflurane (2%) and oxygen (3 L/min) via nose cone (Patterson Scientific anesthesia machine/nose cone). They were placed with a modified cup around the anesthesia system to suction anesthetic waste. Guinea pigs were secured using double sided fabric hook-and-loop fasteners to limit movement of the legs during the 180 min evaluation. During the imaging study vitals measured included heart rate, ECG and respiratory rate. They were not recovered following the iohexol study. During the imaging study vitals measured included heart rate, ECG (3M Red Dot prewired ECG monitoring electrode) and respiratory rate (visually by the technician). Isoflurane anesthesia was adjusted during the 180 min when heart rate increased above 250 beats per minute or respiratory rates exceeded 25 breaths per minute. The same 2 individuals positioned all guinea pigs throughout the entire study.

The time at which each guinea pig received its first assigned IM injection was designated time 0. After time 0, guinea pigs were positioned in the CT machine as described above and a short acquisition scan was completed to ensure correct placement. The first CT scan occurred 10 min following time 0. The entire process from the injection, placement in CT and acquisition took less than 6 min for each guinea pig. A total of 18 CT scans were completed for each animal (a scan every 10 min, for a total of 180 min, following iohexol administration). Once scanning was completed, the guinea pigs were placed at 5% isoflurane to achieve deep anesthesia (verified by a negative toe pinch response) and were euthanized by intracardiac injection of 0.4 mL Sodium Pentobarbital (Sodium Pentobarbital 390 mg/mL, Fatal Plus Vortech Pharmaceuticals) in accordance with the AVMA *Guidelines for Euthanasia of Animals*.[14] Secondary confirmation was an absence of vital signs, including lack of respiration and heart sounds, at 10 min after intracardiac injection of sodium pentobarbital.

CT image acquisition, reconstruction and analysis. As per the previous study,[10] each scan consisted of a single, approximately 4.5 min static frame (80 kV, 500 µA, 98 µm, 360° rotation in 220 steps) on a CT imaging system (Inveon, Siemens Medical Solutions, Knoxville, TN). All CT images were reconstructed into a 3D volume with 512-µm isotropic resolution by using the Inveon Acquisition Workplace version 2.1 software package (Siemens). Hounsfield unit (HU)-calibrated CT data were reconstructed by using the Feldkamp reconstruction algorithm, with a Shepp-Logan reconstruction filter, slight noise reduction, and beam-hardening correction applied.

CT data were analyzed by using image processing software (VivoQuant version 2.5, inviCRO, Boston, MA) and results were reported in terms of mean HU per region of interest (ROI). For CT image analysis, the distribution and intensity of the iohexol signals were examined to determine tissue distribution over time for each volume group. Signal intensity was determined by quantifying mean HU within each ROI over time. Initial injection-volume ROI were automatically and objectively defined by placing a seed point within the hyperintense injection site on the first (time, 10 min) image for each guinea pig and then letting the analysis software define the borders of the injection volume by using a lower-limit thresholding algorithm that includes voxels into the ROI when they are contiguous with the seed point, and have a value at or above 2 times muscle background plus 2 standard deviations (SD). Pelvic limb ROI were manually drawn on the last image in the time series (180 min) to include the hyperintense region of distributed iohexol within the pelvic limb and surrounding tissues. Pelvic limb ROI were drawn by an experienced data analyst. Once completed, ROI for the 10- and 180-min time points were applied to each time point in the 18-image series, and average HU intensity was determined for each ROI over the duration of the study. Leakage of HU-intense iohexol away from the initial intramuscular injection site was measured as increases in mean HU intensity in the surrounding pelvic limb ROI over time.

The determination of intramuscular leakage was computed as previously described in the literature.[10] As in the previous study,[10] the methodology used to assess leakage from the initial injection site was to define ROI for both the initial injection site (time, 10 min; illustrated as the green ROI in Figure 3) and the final surrounding pelvic limb ROI (time, 180 min; the pink ROI in Figure 3). To measure leakage, the final ROI excluded the initial ROI HU value, to quantify changes in HU intensity across time. Increases in CT-measured HU value in the final ROI were interpreted as leakage of HU-intense iohexol from the injection site (blue ROI) into the surrounding tissue. For the larger injection volumes (1000 and 1500 µL), leakage from the injection site into adjacent tissues created pockets. As in previous studies[10] the best way to capture any leakage out of the initial intramuscular injection site was to create a final pelvic limb ROI that was based on the last time point (t = 180 min) for every animal, regardless of injection volume. Drawing the pelvic limb ROI on the final image was necessary to capture the pockets that did not exist at the earlier time points and that swelled only as HU-intense iohexol leaked from the injection site and into the adjacent surrounding tissue (Figure 3). The pelvic limb ROI that was drawn according to the 180-min image for each guinea pig was then applied to all of the other imaging time points to capture any leakage into this space throughout the study.

Clinical leakage assessment. Once images were reconstructed, a slide deck consisting of the 18 serial CT scans was created for each guinea pig leg and placed in a time loop to act as a visual means to determine iohexol tissue distribution in and away from the initial site of injection. The 18 CT scans were sequentially compiled to compress the 180-min timeline of iohexol tissue distribution into 18 static images that were played in rapid succession. Each slide deck was placed in a time loop, set to cycle repeatedly from 10 to 180 min over the course of 2 s, effectively creating a video image. Time-looped slide decks were randomized and compiled into a single, comprehensive slide deck. To test interrater variability in assessing the degree of iohexol leakage or tissue distribution in the injection site, the comprehensive slide deck was presented to 34 blinded veterinarian raters for scoring according to defined criteria (Figure 4). Raters received a visual example of each score by placing synchronized time-looped slide decks side by side for comparison (one time-looped slide deck for each score, 0 to 4). Raters scored all 54 time- looped slide decks at their own

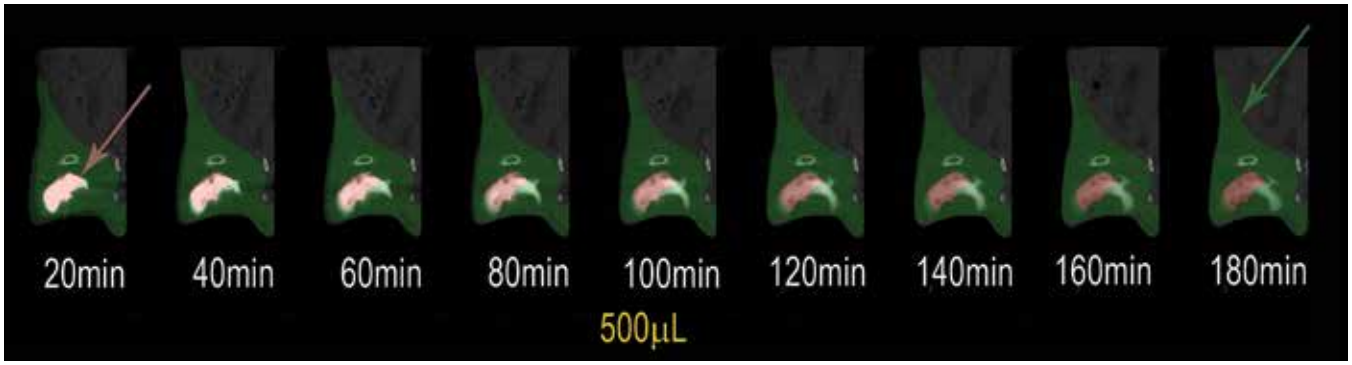

Figure 3. To quantify and measure leakage from the site of injection, we used a region of interest (ROI) analysis template. A 3D ROI image was drawn for the initial injection (pink arrow; pink ROI) at the beginning of each initial scan (10 min). Another 3D image was then created to cover over the entire pelvic limb region (green arrow; green ROI) at the last scan (180 min). During this analysis the volume of final ROI (green pelvic limb ROI) did not include that of the initial injection volume (pink ROI). The dispersion by time of the iohexol at the site of the injection is associated with increasing average Hounsfield unit intensity in the pelvic limb (green) ROI over time.

0 - Contrast agent does not appear to move beyond initial injection site and dissipates rapidly.
1- Minimal expansion of contrast agent within the muscle tissue but little or no contrast leaks from muscle; rapid dissipation.
2 - Minimal expansion of contrast agent within the muscle tissue and minimal contrast leaks from muscle into intermuscular space; rapid dissipation.
3 - Moderate leakage of contrast agent away from the initial injection site that extends to intermuscular space and subcutaneous tissues; slower dissipation.
4 - Severe leakage of contrast agent from initial injection site that rapidly extends into the intermuscular space and subcutaneous tissues; extremely slow dissipation.

Figure 4. Clinical leakage assessment scores and their descriptions were used to score 54-time looped slide decks (52 original, 2 repeats)

pace, but completed scoring the comprehensive slide deck in a single session.

Statistical analysis. Guinea pigs were randomized to receive one of the 5 possible pairs of injection volumes. The pairings were selected to maximize statistical power while minimizing the total volume of iohexol administered to each animal. The association between the percent decrease in HU concentration at the injection site ROI 30, 60, and 90 min post injection was analyzed by linear mixed-effect model. The effect of the contralateral volume was analyzed under a similar statistical model, by comparing the decrease at 60 min across each of the possible contralateral volumes. The models included a compound symmetric correlation structure to adjust for the pairing of treatments on each animal. Image scores supplied by blinded veterinarians were summarized as the frequency and percentage of all scores having each of the 5 (0 to 4) possible values. The mean scores at each injection volume were estimated and compared by a repeated measures ANOVA, along with an estimate of the class correlation, as described by Shrout and Fleiss.[21,25] Each veterinarian scored all available images. All analysis was implemented in SAS version 9.4.[21] Statistical significance was set at $P < 0.05$ throughout.

Clinical observations and endpoint euthanasia. In the second experiment, clinical signs of pain were determined from previous publications describing the development of endpoints for other species, and surgical endpoints for guinea pigs.[4,8,15,24] Because we were attempting to identify appropriate endpoints, we chose a set score for potential euthanasia. If an animal scored a minimum of 10 from a combination of parameters *(appearance, eye opening, breathing, in-cage activity, mucous membrane, activity during handling, and bodyweight loss)*, euthanasia was performed based on discussion between the attending veterinarian and investigator (Figure 1). Guinea pigs were observed twice a day with greater than 8 h between observations for 3 d post injection. One experienced animal technician who was trained on the scoring chart (Figure 1) performed all 8 consecutive observations. Seventy-two h post injection, all of the guinea pigs had reached the study end point and were euthanized. Euthanasia was performed by placing the the guinea pigs into an Integrated Multi-Patient Anesthetic Center [IMPAC6], (Vet Equip, Piney River, VA) with the Isoflurane vaporizer set to deliver a 5% mixture of isoflurane with oxygen into the chambers to achieve a deep anesthetic plane. The appropriate depth of anesthesia was confirmed by lack of a response totoe pinch, and euthanasia was performed by administering a dose of 0.4 mL of Sodium Pentobarbital via intracardiac injection. Once the death of the animals was confirmed, both legs were sectioned from the body and placed in a vial filled with 10% formalin for fixation and processing prior to pathologic analysis.

Pathology evaluation. In the second study, the pathologist assessed the pathologic changes caused by the injection of different volumes of sodium chloride into the semimembranosus muscle, compared with the contralateral control leg in guinea pigs. 25 guinea pigs (12 female and 13 males) had varying IM injections volumes (150 to 1500 µL). Injections were randomized between left and right and volumes within groups. All collected tissues were fixed in 10% neutral buffered formalin for at least 21 d. Once the tissues were fixed, they were trimmed, processed, embedded in paraffin, cut at 5 to 6 µm and stained with hematoxylin and eosin (H and E) for histopathology analysis. Tissues entering the histology laboratory were subsequently

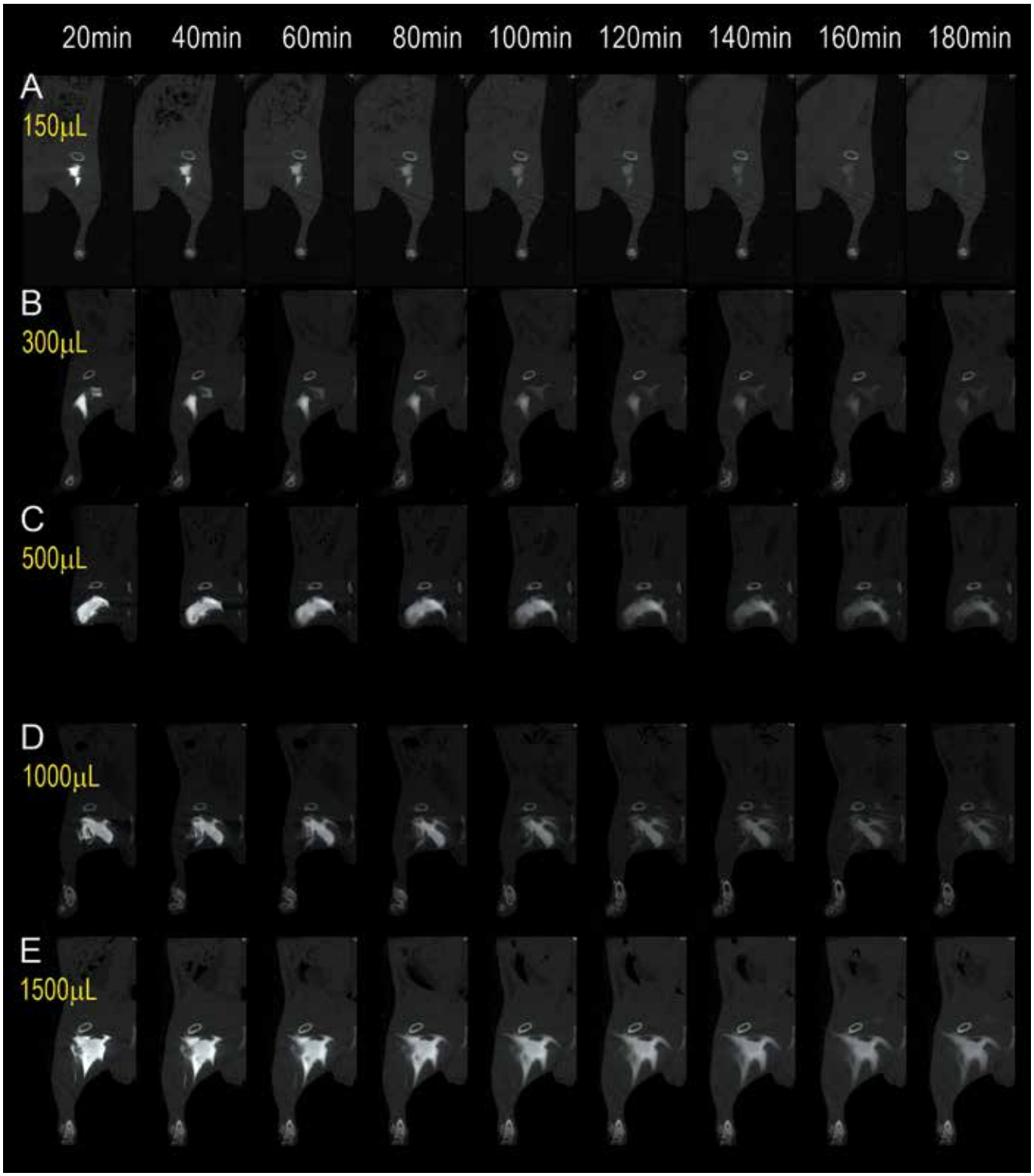

Figure 5. Iohexol (240 mg/mL) was injected intramuscularly into the semimembranosus muscle of guinea pigs and the tissue distribution was evaluated every 10 min up to 180 min. In the (A) 150 µL and (B) 300 µL, the injected iohexol appears to remain within the intramuscular space throughout the study. In the (C) 500 µL group there does appear to be slight leakage into the extramuscular space. However, once levels pass 500 µL to (D) 1000 µL and (E) 1500 µL a considerable portion of the intramuscularly injected iohexol appears to leak considerably throughout the leg. Representative animals from each group are shown.

assigned an accession number, coded by left or right leg and animal microchip ID.

Results

Observational pain assessment and scoring. There was no statistically significant association between volume and increased pain or decreased activity. No signs of pain, reduction in eating or other normal activity, or irritation at the injec-

Table 1. Percent reduction in concentration at the injection site ROI following IM injection with iohexol. We calculated the initial ROI, measured through HU, and then at 30/60/90 min we calculated how much the HU has decreased as a measure of absorption. There was a trend toward less absorption at greater injection volumes.

	Injection volume (µL)				
Time post injection	150	300	500	1000	1500
30 min.	27.9 (32.5, 23.0)	19.7 (23.9, 15.3)	23.4 (28.0, 18.5)	13.8 (19.0, 8.3)	12.8 (20.5, 4.4)
60 min.	54.3 (58.2, 50.0)	42.1 (46.1, 37.8)	48.8 (53.0, 44.2)	31.0 (36.4, 25.1)	21.9 (31.8, 10.7)
90 min.	67.1 (71.3, 62.4)	55.9 (60.2, 51.1)	61.9 (66.5, 56.8)	44.8 (51.4, 37.3)	42.1 (52.6, 29.2)

Displayed values indicate mean (95% CL) of the percent reduction in the injection site ROI concentration post injection.

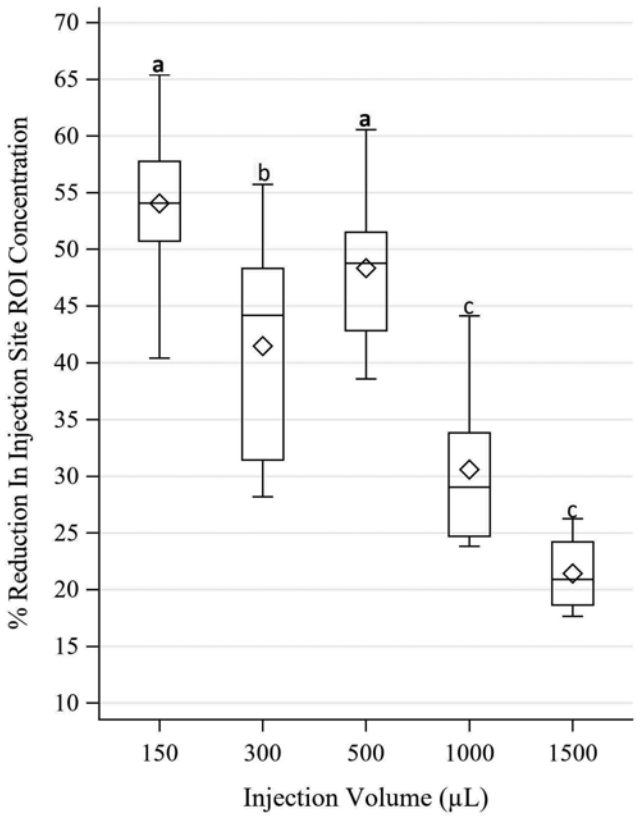

Figure 6. The percent reduction in the injection site ROI concentration (HU/Voxel) was measured 60 min post intramuscular iohexol injection. Groups are significantly different ($P < 0.05$ by linear mixed effect model) only where the corresponding letter designations differ. The 150 µL group differed from the 300, 1000 and 1500 µL groups ($P = 0.0004$, $P < 0.0001$, and $P < 0.0001$, respectively), as did the 500 µL group ($P = 0.0364$,, $P < 0.0001$, and $P < 0.0001$, respectively). The 300 µL group also differed from both the 1000 µL and 1500 µL groups ($P = 0.0034$ and $P = 0.0008$, respectively). Diamond indicates mean value, line indicates median, box indicates interquartile range, and whiskers extend to minimum and maximum.

tion site were noted, despite the varying injection volumes. Following randomized assigned injections of 0.9% NaCl, all guinea pigs were observed twice a day and weighed once a day by an experienced technician using an assigned ethogram (Figure 1). They were also assessed for licking, irritation or biting at the assigned injection site. Only one guinea pig scored a cumulative score of 1 on its clinical observation. All other guinea pigs scored a 0 throughout the 72 h observation window. The animal that scored a one was based on a 7% reduction in weight in the first 24 h (368 to 343.4 grams) which could have been based on a reduction in eating or an initial weight error.

Table 2. The percent reduction in concentration at the injection site ROI 60 min post injection by contralateral injection volume. Animals were injected IM in each leg with varying volumes of iohexol. The purpose of this table was to evaluate the relationship between different intramuscular injections placed into the contralateral leg of the same guinea pig. A slight inverse relationship between absorption rate and contralateral injection volume was found.

		Contralateral injection volume (µL)				
Time point (min.)	Injection volume (µL)	150	300	500	1000	1500
60	150		57.4			50.6
	300	48.6		36.2[a]	39.9	
	500		49.4		48.3	
	1000		27.9	33.2		
	1500	21.5				

Displayed values indicate mean of the percent reduction in the injection site ROI concentration 60 min post injection.
[a] Significant difference from 150 µL contralateral volume.

Pathology. Histopathology related to the IM injection was detected in 10/25 animals; histologic lesions included muscle degeneration and hemorrhage (5/10), adipose inflammation (3/10), muscle degeneration only (1/10), and hemorrhage in adipose tissue (1/10). The remaining 15 animals display either no findings (10/15) or nonexperimental related chronic myositis (5/15). Muscle degeneration was characterized as sarcoplasmic swelling, hypereosinophilic staining, loss of striations, absence of nuclei, fragmentation and/or vacuolation of the sarcoplasm. Muscle degeneration was limited to a focal or locally extensive region. Hemorrhage was commonly found in these areas of muscle degeneration with the one expectation, which displayed muscle degeneration alone. Acute inflammation was the second category of lesions commonly detected (3/10) associated with IM injections. Inflammatory sites were infiltrated by minimal numbers of macrophages, lymphocytes and neutrophils, and inflammatory infiltrate was confined to adipose tissue rather than penetrating the muscle. For one animal, extensive hemorrhage with erythrophagocytosis and hemosiderin-laden macrophages was present in adipose tissue.

Qualitative assessment of intramuscular iohexol leakage and tissue distribution. We injected 150, 300, 500, 1000 and 1500 µL of iohexol (240 mg/mL) into the semimembranosus muscle of guinea pigs and evaluated tissue distribution over a 3 h (180 min) time course. In each leg given 150- and 300-µL, the iohexol appeared to remain in the intramuscular space and was absorbed centrally over time (Figure 5). When the injection volume was equal to or greater than 500 µL, substantial amounts of the solution appeared to leak from the injection site into the surrounding tissues.

Quantitative assessment of intramuscular iohexol leakage and tissue distribution. We used 3D ROI to quantitatively assess differences in absorption and leakage of injected iohexol from

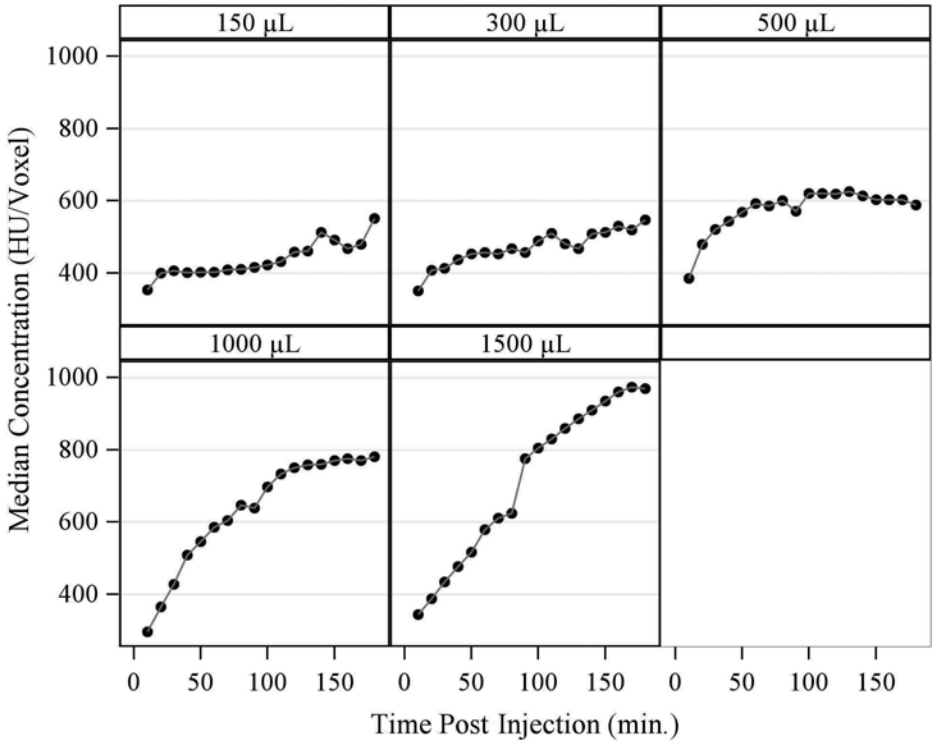

Figure 7. Following intramuscular iohexol injection, the concentration (HU/Voxel) in the whole leg ROI, excluding the injection site, was measured over time. A trend toward greater spread beyond the initial injection site ROI at higher injection volumes was observed. The rate at which iohexol was absorbed showed only a small association with animal weight at lower injection volumes. The effect of animal weight was statistically significant at the 150 µL injection volume ($P < 0.05$), but not at any other injection volume.

the injection site of each guinea pig. Comparing 10 min from the initial injection time through 180 min, we were able to assess the percent reduction in concentration at the injection site. There was a statistically significant ($P < 0.05$ by log-log linear regression) trend toward less absorption at greater injection volumes 30, 60 and 90 min (Table 1).

The percent reduction in iohexol concentration at the injection site 60 min after IM injection was also evaluated. Increased injection volumes were associated with a statistically significant ($P < 0.05$ by log-log linear regression) decrease in the rate of absorption from the injection site (Figure 6).

In this study, the guinea pigs were injected IM in each leg with differing volumes of iohexol. Using both legs for evaluation in this manner did have some effects on absorption time (Table 2). A slight inverse relationship between the absorption rate and contralateral injection volume was found. For example, in those legs injected with 300 µL, contralateral volumes of 150 µL were associated with a mean reduction in HU concentration of 48.6%, while contralateral injection with 500 µL resulted in a mean reduction of only 36.2%, an effect which was statistically significant ($P < 0.05$).

Leakage of HU-intense iohexol from the intramuscular injection site was measured as increases in mean HU intensity in the surrounding pelvic limb ROI over time. Area under the curve analysis showed that as the initial intramuscular injection volume increased from 150 to 1500 µL, mean HU intensity in the pelvic limb ROI increased over time (Figure 7). Although mean HU intensity in the pelvic limb increased slightly when the injected volume increased from 150 to 500 µL, the AUC for both volumes were similarly flat, indicating that the iohexol remained nearly exclusively intramuscularly at the injection site and did not markedly leak into the surrounding tissue over time.

Clinical assessment of intramuscular iohexol leakage and tissue distribution. We calculated the mean and standard error of the scores for each volume group, to quantify the subjective visual scores for the 54 time-looped slide decks that were scored by 34 blinded veterinarian raters. As injection volume increased the score assigned by the raters also increased (Table 3). There was a significant correlation between the log of the injection volume and the score ($P < 0.05$). The mean score increased 0.97 points (95% Confidence Interval (CI) 0.886 to 1.052) for each doubling of the injection volume. Interrater reliability was quantified by intraclass correlation (ICC) and found to be 0.71, a value consistent with good agreement between raters. Based on these findings, volumes below 500 µL scored 2 or below, indicating minimal distribution and leaks of the contrast agent. When the volume exceeded 500 µL, scores hit levels of greater than a score of 3 (Figure 4), indicating moderate leakage and distribution of the contrast agent.

Discussion

In the experiment examining tissue distribution of iohexol injected IM, our findings suggest that the optimal volume capacity for intramuscular injection of guinea pigs in the semimembranosus muscle is less than 500 µL (Table 3 and Figures 6 and 7). The most rapid IM absorption occurred at 300 µL. Comparing results of CT analysis and the clinical leakage assessment, the volume displaying the least expansion and most rapid absorption would be 300 µL, as noted in the 3rd edition of *Laboratory Animal Medicine*.[28] Evaluating the results of the clinical leakage assessment, (Table 3) 52% of evaluators scored the 500 µL volume as a 2 or below. An effective injection is one that is absorbed and has minimal leakage outside of the intended injection site. A score of 2 is defined as minimal expansion of contrast agent within the muscle tissue and minimal contrast

Table 3. Blinded veterinarians ($n = 34$) scored time looped slide decks ($n = 54$) according to the criteria of Figure 4. There was a significant correlation between the log of the injection volume and score ($P < 0.05$). The mean score increased 0.97 points (95% CL 0.886 to 1.052) for each doubling of the injection volume. Interrater agreement was quantified by intraclass correlation (ICC) and found to be 0.71, a value consistent with good agreement between raters.

	Injection volume (µL)					
Score	150	300	500	1000	1500	Total
0	162 (48%)[b]	47 (9%)	12 (3%)	0 (0%)	1 (1%)	222 (13%)
1	137 (40%)	228 (45%)	37 (10%)	6 (2%)	0 (0%)	408 (23%)
2	35 (10%)	190 (37%)	145 (39%)	45 (12%)	1 (1%)	416 (24%)
3	5 (1%)	41 (8%)	153 (41%)	177 (47%)	20 (12%)	396 (22%)
4	1 (0%)	4 (1%)	27 (7%)	146 (39%)	148 (87%)	326 (18%)
Mean(Stderr), Score	0.66 (0.122)	1.46 (0.106)	2.39 (0.119)	3.26 (0.117)	3.80 (0.158)	
Slope (95% CL)[a]						0.97 (0.886, 1.052)
Inter-Rater Agreement (95% CL)[c]						0.71 (0.624, 0.792)

Mean standard errors, and regression parameters are estimated under a linear mixed effects model.
[a] Mean Change Per 2 fold increase in injection volume
[b] Number and percentage of all ratings.
[c] The class correlation measures interrater agreement, values near 1 indicate good agreement between raters.

leaks from muscle into intermuscular space; rapid dissipation. For this evaluation, a score of 2 or below met our criteria for an effective injection. Thus, if volumes lower than 500 µL (that is, 400 µL) were evaluated for injection into the semimembranosus muscle, we may have identified the optimal intramuscular volume, rather than concluding 300 µL was the best volume. We could also conclude that as the injection volume increased, the amount of solution that redistributed to the surrounding tissues increased.

The CT evaluation could have a number of potential confounding variables. For one, iohexol is a hypertonic solution which could affect absorption amount and distribution. One of the minor variables found during this experiment (Table 2) was a difference in contralateral injection volumes. The decision to inject both legs was based on Russell and Burch's principle of reduction.[20] We did note a minor difference in absorption, based on higher injection volumes of the adjacent leg. A slight inverse relationship between absorption rate and contralateral injection volume was found (Table 2). This could potentially be of importance if an investigator wanted to use the contralateral leg for additional injections. Iohexol has also been known to cause acute kidney failure, and this factor could play a role in impeding absorption.[1]

In experiment 2, the guinea pigs did not appear to be in any increased pain or distress based on the observations. One would expect with such a large volume (1000 to 1500 µL) might cause a weight loss due to decreased eating and drinking, lameness, or irritation at the injection site. Based on previous research, guinea pigs are known to hide signs of distress through 'conservation withdrawal'.[8] One major concern in our assessment of pain was that the guinea pigs were sedated prior to injection. Potentially, the pain induced by larger volumes may be maximal at the time of injection when it could not be assessed due to sedation. The decision was made to sedate these animals to ensure the accuracy of the injectate within the semimembranosus muscle and to allow subsequent pathologic evaluation of the leg.

Histopathologic evaluation of the hematoxylin and eosin slides reveals no lesions specifically caused by the varying volumes of sodium chloride injected into the semimembranosus muscle. Findings such as muscle degeneration, inflammation, or hemorrhage (or both) were predominantly associated with needle damage from injections (Figure 8). In addition, 10 animals from various volume groups had no detectable pathology findings. Our findings are similar to findings in research performed

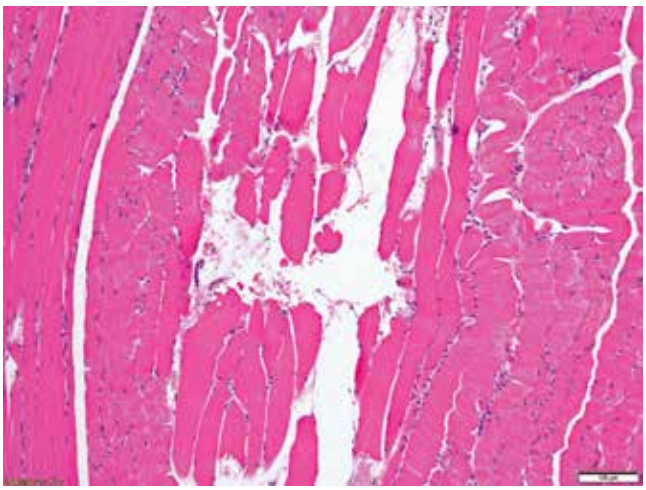

Figure 8. Needle tract damage of the right thigh, skeletal muscle: Focal area of myofiber sarcoplasm swelling and hypereosinophilic staining (muscle degeneration) with fibrin and hemorrhage. Findings were from a female guinea pig who received 300 µL of NaCl in its right hind leg. H and E.

in other laboratory rodents and rabbits.[29] Increased saline volume may result in increased edema separating myofibers bundles and interstitial space.[29] However, measuring edema with standard pathology methods is difficult and subjective. We also observed chronic spontaneous myositis in the hind limbs of guinea pigs, as previously reported, with no evidence of etiology upon histopathology evaluation.[22]

Our iohexol study supported the hypothesis that as the injection volume increased, so would the risk of distribution of the injectate beyond the intended target tissue and of delayed systemic absorption from the muscle. The saline study demonstrated that a majority of the tissue damage was needle tract related. However, needles are known to cause only minor damage, as shown in previous literature.[29] Larger volumes of injectate were not associated with greater pathology or pain. This finding did not support our hypothesis that an increase in pathology and pain would occur with increased volume.

One major element that was not evaluated was the role played by the physical properties of the injectate such as viscosity, pH, temperature, or even hypertonicity. These properties have been known to influence damage to the surrounding tissue, but also

affect the rate of absorption.[16,26,30] We may have concluded that 300 µL, or any volume less than 500 µL, is ideal for IM injection, but that is based on a neutral formulation of injectate.

One recommendation for a future study would be to inject only one leg during CT to solidify any potential confounding effect of the contralateral injections. Those results could be compared with the findings reported here. As for pathology, an excellent experiment would be to evaluate diverse needle types, since this factor appears to have a major pathologic effect on muscle tissue in past and current research.[22] In addition, to improve pain evaluations, we would not sedate the animal to allow the evaluation any immediate pain responses. Using commonly used injectates with varying properties, like increased pH, would also provide information regarding optimal injection volumes for both guinea pigs and other commonly used research animals.

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References

1. **Almén T.** 1990. Contrast media: the relation of chemical structure, animal toxicity and adverse clinical effects. Am J Cardiol **66:**F2–F8. https://doi.org/10.1016/0002-9149(90)90634-D.
2. **Barthold SW, Griffey SM, Percy DH.** Guinea pig, p 217–251. Pathology of laboratory rodents and rabbits, 4th ed. Ames (IA): Blackwell Publishing.
3. **Cashman KA, Wilkinson ER, Wollen SE, Shamblin JD, Zelko JM, Bearss JJ, Zeng X, Broderick KE, Schmaljohn CS.** 2017. DNA vaccines elicit durable protective immunity against individual or simultaneous infections with Lassa and Ebola viruses in guinea pigs. Hum Vaccin Immunother **13:**3010–3019. https://doi.org/10.1080/21645515.2017.1382780.
4. **Collymore C, Kent L, Ahn SK, Xu W, Li M, Liu J, Turner PV, Banks EK.** 2018. Humane endpoints for guinea pigs used for *Mycobacterium tuberculosis* vaccine research. Comp Med **68:**41–47.
5. **Crockett C, Ha R.** 2010. Data collection in the zoo setting, emphasizing behavior, p 386–406. In: Kleiman DG, Allen ME, Thompson KV, editors. Wild mammals in captivity: principles and techniques, 2nd ed. Chicago (IL): Chicago Press.
6. **Diehl KH, Hull R, Morton D, Pfister R, Rabemampianina Y, Smith D, Vidal JM, van de Vorstenbosch C, European Federation of Pharmaceutical Industries, Association and European Centre for the Validation of Alternative Methods.** 2001. A good practice guide to the administration of substances and removal of blood, including routes and volumes. Journal of applied toxicology. JAT **21:**15–23.
7. **Drugs.com.** [Internet]. 2019. Iohexol dosage. [Cited 16 September 2019]. Available at: https://www.drugs.com/dosage/iohexol.html.
8. **Dunbar ML, David EM, Aline MR, Lofgren JL.** 2016. Validation of a behavioral ethogram for assessing postoperative pain in guinea pigs (*Cavia porcellus*). J Am Assoc Lab Anim Sci **55:**29–34.
9. **Gad SC, Spainhour CB, Shoemake C, Pallman DR, Stricker-Krongrad A, Downing PA, Seals RE, Eagle LA, Polhamus K, Daly J.** 2016. Tolerable levels of nonclinical vehicles and formulations used in studies by multiple routes in multiple species with notes on methods to improve utility. Int J Toxicol **35:**95–178. https://doi.org/10.1177/1091581815622442.
10. **Gehling AM, Kuszpit K, Bailey EJ, Allen-Worthington KH, Fetterer DP, Rico PJ, Bocan TM, Hofer CC.** 2018. Evaluation of volume of intramuscular injection into the caudal thigh muscles of female and male BALB/c mice (*Mus musculus*). J Am Assoc Lab Anim Sci **57:**35–43.
11. **Institute for Laboratory Animal Research.** 2011. Guide for the care and use of laboratory animals, 8th ed. Washington (DC): National Academies Press.
12. **Klueber KM, Ontell M.** 1984. A new approach to intramuscular placement of horseradish peroxidase. Muscle Nerve **7:**127–129. https://doi.org/10.1002/mus.880070207.
13. **Leach MC, Allweiler S, Richardson C, Roughan JV, Narbe R, Flecknell PA.** 2009. Behavioural effects of ovariohysterectomy and oral administration of meloxicam in laboratory housed rabbits. Res Vet Sci **87:**336–347. https://doi.org/10.1016/j.rvsc.2009.02.001.
14. **Leary S, Underwood W, Anthony R, Cartner S, Corey D, Grandin T, Greenacre CB, Gwaltney-Bran S, McCrackin MA, Meyer R, Miller D, Shearer J, Yanong R.** 2020. AVMA guidelines for the euthanasia of animals: 2013 edition.Schaumburg (IL): American Veterinary Medical Association.
15. **Nunamaker EA, Artwohl JE, Anderson RJ, Fortman JD.** 2013. Endpoint refinement for total body irradiation of C57BL/6 mice. Comp Med **63:**22–28.
16. **Okumura K, Sezaki H, Kakemi K.** 1972. Absorption of drugs from the skeletal muscle of the rats. 4. Absorption of cationic drugs from the muscle. Chem Pharm Bull (Tokyo) **20:**1607–1611. https://doi.org/10.1248/cpb.20.1607.
17. **Pam N.** [Internet]. 2013. Conservation withdrawal. [Cited 16 September 2019]. Available at: https://psychologydictionary.org/conservation-withdrawal/.
18. **Pingel J, Nielsen MS, Lauridsen T, Rix K, Bech M, Alkjaer T, Andersen IT, Nielsen JB, Feidenhansl R.** 2017. Injection of high dose botulinum-toxin A leads to impaired skeletal muscle function and damage of the fibrilar and non-fibrilar structures. Sci Rep **7:**14746. https://doi.org/10.1038/s41598-017-14997-3.
19. **Rouve E, Lakhal K, Salmon Gandonniere C, Jouan Y, Bodet-Contentin L, Ehrmann S.** 2018. Lack of impact of iodinated contrast media on kidney cell-cycle arrest biomarkers in critically ill patients. BMC Nephrol **19:**1–7. https://doi.org/10.1186/s12882-018-1091-2.
20. **Russell WMS, Burch RL.** 1959. The principles of humane experimental technique. Wheathampstead (UK): Universities Federation for Animal Welfare
21. **SAS.** 2020**.** [Internet]. Version 9.4. [Cited 18 July 2020]. Available at: https://www.sas.com/en_us/software/sas9.html
22. **Saunders LZ.** 1958. Myositis in guinea pigs. J Natl Cancer Inst **20:**899–903.
23. **Sedgwick CJ, Martin JC.** 1994. Concepts of veterinary practice in wild mammals. Vet Clin North Am Small Anim Pract **24:**175–185. https://doi.org/10.1016/S0195-5616(94)50009-3.
24. **Sharpe SA, McShane H, Dennis MJ, Basaraba RJ, Gleeson F, Hall G, McIntyre A, Gooch K, Clark S, Beveridge NE, Nuth E, White A, Marriott A, Dowall S, Hill AV, Williams A, Marsh PD.** 2010. Establishment of an aerosol challenge model of tuberculosis in rhesus macaques and an evaluation of endpoints for vaccine testing. Clin Vaccine Immunol **17:**1170–1182. https://doi.org/10.1128/CVI.00079-10.
25. **Shrout PE, Fleiss JL.** 1979. Intraclass correlations: Uses in assessing rater reliability. Psychol Bull **86:**420–428. https://doi.org/10.1037/0033-2909.86.2.420.
26. **Smiler KL, Stein S, Hrapkiewicz KL, Hiben JR.** 1990. Tissue response to intramuscular and intraperitoneal injections of ketamine and xylazine in rats. Lab Anim Sci **40:**60–64.
27. **Svendsen O.** 2005. Ethics and animal welfare related to in vivo pharmacology and toxicology in laboratory animals. Basic Clin Pharmacol Toxicol **97:**197–199, author reply 200–191. https://doi.org/10.1111/j.1742-7843.2005.pto_letter_974.x.
28. **Talcott MR, Akers W, Marini RP.** 2015. Techniques of experimentation, p 1201–1262. Chapter 25. In: Fox JG, Anderson LC, Otto GM, Pritchett-Corning KR, Whary MT editors. Laboratory animal medicine, 3rd ed. Boston (MA): Academic Press. https://doi.org/10.1016/B978-0-12-409527-4.00025-0

29. **Thuilliez C, Dorso L, Howroyd P, Gould S, Chanut F, Burnett R**. 2009. Histopathological lesions following intramuscular administration of saline in laboratory rodents and rabbits. Exp Toxicol Pathol **61:**13–21. https://doi.org/10.1016/j.etp.2008.07.003.
30. **Wagner JG.** 1969. Aspects of pharmacokinetics and biopharmaceutics in relation to drug activity. Am J Pharm Sci Support Public Health **141:**5–20.
31. **Wickstrom AM.** 2007. Exotic pet behavior — birds, reptiles, and small mammals. Can Vet J **48:**1296–1296.