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Research funding by the ACVS Foundation

Safety and feasibility of near-infrared fluorescence imaging with inhaled indocyanine green to assess the pulmonary parenchyma in six normal dogs

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Funding information

American College of Veterinary Surgeons Foundation, Grant/Award Number: 5868

Abstract

Objective: To assess the safety and feasibility of near-infrared fluorescence (NIRF) imaging with inhaled indocyanine green (ICG) to visualize normal pulmonary parenchyma in dogs, report clinically useful information regarding dosage and timing of administration, and to evaluate effects on pulmonary function and tissue histology.

Study design: Preclinical exploratory study.

Animals: Six purpose-bred dogs.

Methods: Dogs undergoing thoracoscopy were randomly assigned to receive inhaled ICG at 0.1, 0.5, or 1.0 mg/kg. Subjective fluorescence was recorded, images were saved to analyze quantitative fluorescence, and a lung biopsy was obtained. Clinical assessment of respiratory function was performed for 14 days. Peripheral lung biopsies were repeated 6 months later.

Results: Inhalation of ICG resulted in real-time NIRF of pulmonary parenchyma in all dogs. Administration time ranged from 1 to 10 min and mean time to first fluorescence was 1.5 min. Peak subjective fluorescence was strong in dogs receiving 0.5 and 1.0 mg/kg, and moderate in dogs receiving 0.1 mg/kg. Peak quantitative fluorescence was positively correlated with dose, with a three-fold and 10-fold increase above baseline 20 min after administration of 0.5 and 1.0 mg/kg doses, respectively. No histologic abnormalities or clinical evidence of respiratory compromise following inhalation of ICG were observed.

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Conclusion: Inhaled ICG provided rapid intraoperative fluorescence of normal pulmonary parenchyma, was clinically feasible, and was safe in normal dogs at 0.1–1.0 mg/kg.

Clinical significance: Inhaled ICG produced rapid pulmonary fluorescence without detectable adverse effects, supporting further investigation of its effectiveness to identify pulmonary lesions in dogs.

1 | INTRODUCTION

Exploratory thoracoscopy may be used to diagnose intra-thoracic disease and facilitate therapeutic intervention using video-assisted thoracic surgery (VATS) or an open surgical approach.^{1,2} When compared to thoracotomy, exploratory thoracoscopy has been associated with fewer complications, decreased postoperative pain and pleural drainage, and shorter hospitalization time in people.^{3,4} Similarly, dogs undergoing thoracoscopic pericardiectomy had lower postoperative pain scores and reduced serum cortisol concentrations compared to dogs undergoing thoracotomy.⁵

Despite the potential for reduced morbidity, applications of exploratory thoracoscopy in veterinary medicine are relatively limited due to inherent difficulty evaluating all of the pleural surfaces thoracoscopically combined with the inability to perform traditional saline submersion leak testing. Spontaneous pneumothorax poses a particular challenge due to the limitations of advanced imaging to accurately identify pulmonary blebs and bullae to inform surgical planning.⁶ Furthermore, treatment of spontaneous pneumothorax with VATS has been associated with a high rate of conversion to median sternotomy due to inability to identify lesions.⁷

A novel technique using inhaled indocyanine green (ICG) in combination with near-infrared fluorescence (NIRF) imaging was recently described in humans and experimental species to facilitate intraoperative detection of lung tumor margins, detect air leakage following pulmonary resection, and allow precise resection of congenital pulmonary disease.^{8–11} When compared to intravenous administration, inhaled ICG in rabbits resulted in a significantly higher signal to background ratio of lung tumor margins which persisted for 24 h following administration.⁸

Inhaled ICG may facilitate thoracoscopic identification of pulmonary lesions in dogs by providing sustained contrast between areas of diseased and healthy tissue in real time. This has the potential to improve the sensitivity of exploratory thoracoscopy, allow for precise resection of lesions, and expand the applications of VATS in veterinary species; however, clinical application of inhaled ICG

in dogs is precluded by insufficient data regarding safety, feasibility, and dose optimization, warranting further study prior to use in clinical trials.

The objective of this study was to evaluate the feasibility of NIRF imaging with inhaled ICG to visualize normal pulmonary parenchyma in dogs thoracoscopically, determine clinically useful information regarding dosage and timing of administration, and to report acute and delayed effects of inhaled ICG on postoperative pulmonary function and histology. We hypothesized that inhalation of ICG would be clinically feasible, result in real time near-infrared fluorescence of normal pulmonary parenchyma, and have no observable adverse effects in dogs.

2 | MATERIALS AND METHODS

2.1 | Animals

Approval for this study was granted by the Institutional Animal Care and Use Committee at North Carolina State University (#21-466). Six purpose-bred large-breed dogs were included. All dogs were free of overt respiratory disease based on physical examination. All dogs were adopted or retained by the institution as part of the canine teaching cohort following the conclusion of this study. Dogs that were kept by the institution were designated for adoption following a limited term of service.

2.2 | Surgical procedures

All surgical procedures were performed by a board-certified surgeon experienced in thoracoscopy and assisted by a surgical resident. Dogs were premedicated with dexmedetomidine 3 mcg/kg and hydromorphone 0.05 mg/kg IV to facilitate establishment of IV access. Induction of anesthesia was achieved using midazolam 0.2 mg/kg and propofol 3–6 mg/kg IV to effect. Total IV anesthesia was maintained using fentanyl 15–30 mcg/kg/h and propofol 150–250 mcg/kg/min. All dogs were mechanically ventilated with a maximal inspiratory pressure of 20 cmH₂O.

A NIRF imaging system was used for white light and fluorescence image acquisition throughout the procedure (Image1 S, Karl Storz Veterinary Endoscopy, Goleta, California). Dogs were positioned in dorsal recumbency and a paraxiphoid 11 mm cannula (Karl Storz Veterinary Endoscopy) was placed transdiaphragmatically to allow for introduction of a 10 mm rigid endoscope with near-infrared capability (Tipcam1 Rubina 0°, Karl Storz Veterinary Endoscopy). A 6 mm cannula (Karl Storz Veterinary Endoscopy) was placed in the right hemithorax at the seventh intercostal space. Following placement of the first intercostal port, the mediastinum was dissected with 5 mm endoscopic scissors (Karl Storz Veterinary Endoscopy) to facilitate visualization of the medial and lateral surfaces of the right cranial, right middle, right caudal, left cranial, and left caudal lung lobes, and the parietal pleural surfaces. The surface of the accessory lobe was visualized through the mediastinum; however not all surfaces were routinely examined. An 11 mm cannula (Karl Storz Veterinary Endoscopy) was placed in the left hemithorax between the seventh and ninth intercostal space, determined by the surgeon intraoperatively based on each dog's individual anatomy. Exploratory thoracoscopy was performed with use of a blunt probe (Karl Storz Veterinary Endoscopy) and dogs were excluded if gross evidence of pulmonary or pleural disease was identified.

Following exploratory thoracoscopy, the endoscope was positioned such that the medial aspect of the right cranial lung lobe occupied the field of view during peak inspiration. This position was maintained to minimize the effect of distance on the strength of fluorescence during NIRF imaging, and all subsequent assessments of fluorescence were performed during a breath hold at 20 cmH₂O to further limit changes in distance caused by respiratory motion. A solution of ICG 2.5 mg/mL (MP Biomedicals, Solon, Ohio) was prepared by mixing 10 mL of sterile water with 25 mg of ICG. The volume of ICG required to deliver the assigned dose was calculated and added to the well of a pediatric jet nebulizer (Henry Schein, Melville, New York). The nebulizer was positioned between the inspiratory limb of the ventilatory circuit and the endotracheal tube (Figure 1). The endotracheal cuff was partially deflated to protect the lungs from barotrauma and the oxygen supply to the nebulizer was set at 4 L/min; this was determined to be the lowest effective flow rate to produce aerosolization of ICG as observed prior to connection to the inspiratory circuit. The endotracheal cuff was reinflated after administration of inhaled ICG was completed. Fluorescence was evaluated as described below.

Following evaluation of pulmonary fluorescence and image acquisition, a peripheral lung biopsy was obtained from the apex of the right middle lobe using a single



FIGURE 1 Administration of inhaled indocyanine green (ICG). Inhaled ICG was administered using a pediatric jet nebulizer that was attached to the inspiratory limb of the anesthetic circuit. The dog's head is to the left of the image and the anesthetic circuit is to the right.

ligating loop (Surgitie, Covidien, Mansfield, Massachusetts). A fenestrated thoracostomy tube (MILA International, Florence, Kentucky) was placed in the left hemithorax under thoracoscopic guidance prior to routine closure of the port sites. All dogs were recovered from general anesthesia and analgesia was maintained with methadone 0.2 mg/kg IV every 6–8 h as needed for the first 24 h postoperatively and carprofen 2.2 mg/kg orally every 12 h for three days.

Six months later, dogs were anesthetized with the same protocol for second-look thoracoscopy performed as previously described. Gross evidence of pleural or pulmonary pathology was documented and compared to previous exploratory thoracoscopy findings. Peripheral lung biopsies were obtained from the apex of the left and right cranial lung lobes using a surgical stapler (EndoGIA 45 mm with Tri-Staple technology [tan and purple cartridges], Covidien). Recovery and postoperative analgesia were provided using the previously described protocol.

2.3 | Evaluation of fluorescence

An online random number generator was used to assign dogs to receive inhaled ICG doses of 0.1, 0.5, or 1.0 mg/kg, resulting in two dogs per group. Doses were chosen based on previously reported inhaled ICG doses used in mice and rabbits for evaluation of lung tumor margins and in dogs for identification of pleural defects.^{8,9} Feasibility was defined as successful intraoperative delivery of inhaled ICG through the anesthetic circuit and generation of observable pulmonary

fluorescence using NIRF imaging within 20 min of administration.

Prior to inhalation of ICG, near-infrared images of the lung were obtained to serve as a baseline for subsequent quantitative fluorescence assessments. The endoscope was initially left in continuous near-infrared mode and the duration of administration and time to first observable fluorescence for each dose was recorded. Fluorescence was subjectively scored by the surgeon who was blinded to the dose but was aware of time of administration. Subjective assessments of fluorescence were recorded as absent, weak, moderate, and strong beginning immediately prior to administration of ICG and every 2 min thereafter for 20 min total. In between assessments, the camera was toggled between white light and near-infrared modes. All assessments of fluorescence were made in monochromatic mode (grayscale image).

Still images were saved immediately prior to administration of ICG to establish a baseline of fluorescence followed by image acquisition at each recorded subjective time point. These images were used for postprocedural quantitative measurement of pulmonary fluorescence. Image analysis software (Image J, National Institute of Health, Bethesda, Maryland) was used to measure pulmonary fluorescence by reporting the mean brightness of a region of interest (ROI) encompassing the entirety of the medial aspect of the right cranial lung lobe. Fluorescence was reported as counts per pixel in arbitrary units (AU), consistent with previous studies.^{12,13}

Peripheral lung biopsies were evaluated by a board-certified pathologist with a microscopic fluorescent imaging system utilizing an excitation wavelength of 785 nm and emission filter range between 816 and 840 nm (Odyssey M, LI-COR Biotechnology, Lincoln, New England) to determine the spatial distribution of fluorescence within pulmonary tissue.

2.4 | Evaluation of safety

Safety was evaluated based on the absence of clinically relevant adverse events during or following inhalation of ICG including hemodynamic instability, impaired gas exchange, hypoxemia, clinical signs of respiratory compromise, persistent clinicopathologic abnormalities, and histopathologic evidence of pulmonary injury.

Heart rate, mean arterial pressure (MAP), end tidal CO₂ (ETCO₂), and pulse oximetry parameters were monitored during inhalation of ICG and recorded every 5 min. Clinical assessment of respiratory function was assessed via physical examination including respiratory rate and respiratory effort, with pulse oximetry performed during

the initial 48 h. Respiratory rate, respiratory effort, and pulse oximetry were monitored hourly for the first 6 h postoperatively, then every 8 h for two days. Respiratory rate, respiratory effort, appetite, and general attitude were recorded every 12 h for the following 12 days. Thoracostomy tube production was also recorded hourly for the first 6 h postoperatively, then every 8 h until persistent negative suction was achieved. The thoracostomy tube was removed when persistent negative suction was maintained for a minimum of 8 h. Adverse events were considered to include any unfavorable clinical observation noted during or within 14 days of inhalation of ICG.

Baseline complete blood counts and serum chemistries were performed one day prior to surgery on all dogs and were repeated 1-, 7-, and 14-days postoperatively. Hematologic parameters evaluated included white blood cells (WBC), Red blood cells (RBC), platelet enumeration, C-reactive protein (CRP), glucose, serum urea (SU), creatinine, albumin, total bilirubin, alanine aminotransferase (ALT), gamma-glutamyl transferase (GGT), sodium, potassium, chloride, and bicarbonate. Postoperative results were compared to baseline values to assess for acute and delayed systemic changes that may be attributable to inhalation of ICG.

Peripheral lung biopsies obtained from each surgical procedure were placed in 10% neutral buffered formalin, embedded in paraffin, sectioned, and stained with hematoxylin-eosin. Tissue samples were evaluated by a board-certified pathologist to assess for any histologic abnormalities, especially those potentially attributable to inhalation of ICG.

2.5 | Statistical analysis

Descriptive analyses of observed changes in clinical, biochemical, and subjective and quantitative fluorescence are reported for each of the previously described time-points. All variables were evaluated for normality using the Shapiro-Wilk test. Data are presented as mean and SD for data that are normally distributed and median and range for data that are not normally distributed.

Biochemical parameters at each time point were compared using repeated measures ANOVA and post hoc analysis was performed using a Turkey's HSD test. Heart rate, MAP, ETCO₂, and pulse oximetry parameters were compared prior to and immediately following administration of inhaled ICG using the Wilcoxon signed-rank test. Commercially available software (SPSS; IBM, Armonk, New York) was used for data analysis. Statistical significance was determined by values of $p < .05$.

Given the exploratory study design, a power calculation was not feasible as expected effect sizes have not

been previously reported. Six dogs were selected to prospectively generate preclinical data regarding the safety and feasibility of NIRF imaging with inhaled ICG at three clinically relevant doses while minimizing animal use. This study was not powered to detect rare adverse events or to support definitive statistical comparison between dose groups.

3 | RESULTS

Six purpose-bred female intact mixed breed dogs were included. Mean age was 7.8 months (± 46 days) and mean bodyweight was 24.4 kg (± 1.4 kg). Mean surgical time was 63 (± 8) min. Median time to thoracostomy tube removal was 14.5 h (10–19 h).

Each dog underwent initial and second-look thoracoscopy without intraoperative complications. No evidence of gross pleural or pulmonary disease was identified during initial or second-look thoracoscopy.

3.1 | Clinical feasibility

Inhalation of ICG was successful in all dogs and was deemed clinically feasible. Median duration of administration using an oxygen flow rate of 4 L/min was 8.5 min (1.4–15 min), resulting in a median delivered ICG dose of 1.3 mg/min (0.08–1.7 mg/min). The total volume of ICG administered varied in accordance with intended dosage and bodyweight and ranged from 1.0–10.0 mL. Mean time to first fluorescence was 1.5 min (± 1.1 min). The total cost of the nebulizer and ICG was approximately \$77.00 at the time of data collection.

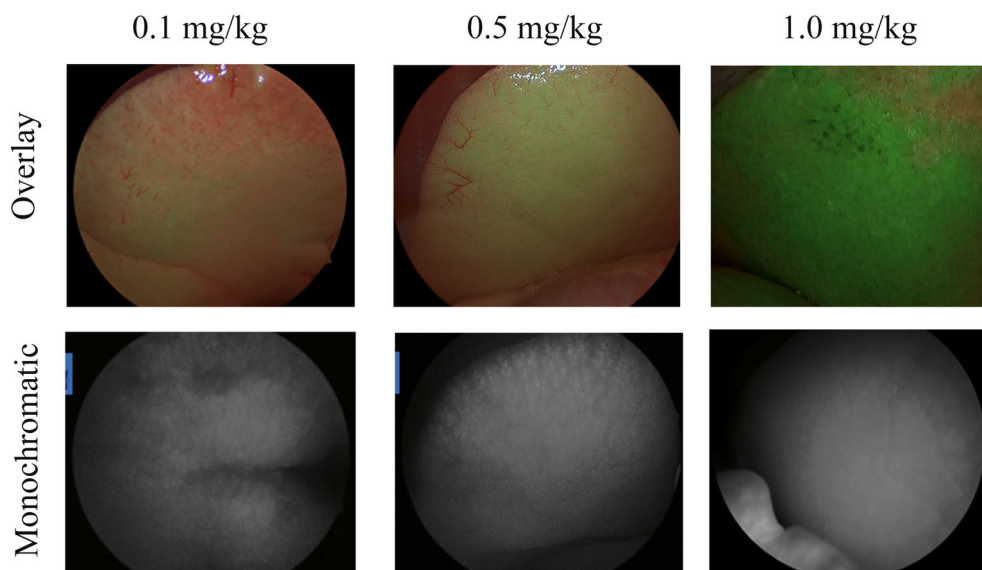
3.2 | Qualitative assessment of fluorescence

Subjective fluorescence of pulmonary parenchyma was rated as moderate to strong in dogs that received ICG doses of 0.5 and 1.0 mg/kg at all assessments (Figure 2). In dogs receiving 1.0 mg/kg of inhaled ICG, peak subjective fluorescence was rated as strong and occurred between 12 and 16 min; fluorescence remained moderately strong at 20 min. In dogs receiving 0.5 mg/kg of inhaled ICG, peak subjective fluorescence was rated as strong and occurred between 0 and 8 min; fluorescence began to subjectively decline by 10 min though it remained moderately strong at 20 min. Peak subjective fluorescence in dogs that received 0.1 mg/kg of inhaled ICG was rated as moderate and occurred between 0 and 8 min; fluorescence then steadily decreased and became weak to absent by 16 min. Fluorescence was noted to be strongest in areas of atelectasis and became weaker once these areas were recruited during breath holds.

3.3 | Quantitative assessment of fluorescence

Peak quantitative fluorescence was positively correlated with ICG dosage and reached a mean of 49 AU, 65 AU, and 82 AU for dogs receiving 0.1, 0.5, and 1.0 mg/kg, respectively. In dogs that received 1.0 mg/kg of inhaled ICG, peak fluorescence occurred between 14 and 20 min. Persistent fluorescence 20 min after inhalation of 1.0 mg/kg ICG remained 10-fold stronger than baseline fluorescence. In dogs that received 0.5 mg/kg of inhaled ICG, peak fluorescence occurred between 2 and 8 min.

FIGURE 2 Representative overlay and monochromatic images of peak fluorescence observed for each dose group after receiving inhaled indocyanine green (ICG). Subjective fluorescence of normal pulmonary parenchyma was rated as moderate to strong in dogs that received ICG doses of 0.5 and 1.0 mg/kg and moderate in dogs that received 0.1 mg/kg.



Persistent fluorescence 20 minutes after inhalation of 0.5 mg/kg ICG remained three-fold stronger than baseline fluorescence. In dogs that received 0.1 mg/kg of inhaled ICG, quantitative fluorescence peaked between 2 and 6 min, then gradually decreased to baseline levels by 20 min.

Microscopic distribution of fluorescence was evaluated from the peripheral lung biopsies obtained from the right middle lung lobe of each dog following administration of inhaled ICG. Fluorescence was distributed predominantly within the pulmonary vessels, with increased fluorescence noted in regions of atelectasis.

3.4 | Safety

No adverse reactions were appreciated during inhalation of ICG and no notable changes in heart rate, MAP, ETCO₂, or pulse oximetry were observed. Heart rate (median preadministration 50 bpm, post-administration 50 bpm [range 48–52 bpm]; $p = .65$), MAP (median preadministration 63 mmHg, post-administration 65 mmHg [range 53–68 mmHg]; $p = .25$), ETCO₂ (median preadministration 47.5 mmHg, post-administration 45 mmHg [range 37–50 mmHg]; $p = .18$), and pulse oximetry parameters (median preadministration 100%, post-administration 100%; range 99%–100%) were not significantly different

prior to and immediately following inhalation of ICG in any dosage group.

All dogs remained eupneic (RR <36 breaths per min with normal effort) and thoracic auscultation was unremarkable for each dog during 14 days of postoperative monitoring. All dogs were normoxemic (SpO₂ > 95%) at each evaluation performed within 48 h of inhalation of ICG. Offered rations were consumed normally throughout the study period and no changes in general attitude were observed. One dog was diagnosed with a superficial surgical site infection at one of the port sites seven days postoperatively which resolved with systemic antimicrobials (Clavamox, Zoetis, Kalamazoo, Michigan) and warm compressing. No additional adverse events were observed during the study period.

Biochemical data are presented prior to and 1-, 7-, and 14-days following inhalation of ICG (Table 1). C-reactive protein was significantly elevated 1 day postoperatively as compared to preoperatively ($p < .01$); the remainder of biochemical parameters evaluated were not significantly affected. Elevations in CRP normalized in all dogs by 14 days postoperatively. No concurrent clinical signs were observed relatable to the elevated CRP.

Peripheral lung biopsies obtained immediately following inhalation of ICG and six months later did not reveal any histologic differences within or between animals. The pulmonary cells appeared unaffected by inhalation

TABLE 1 Biochemical data for dogs prior to and 1-, 7-, and 14-days following administration of inhaled ICG.

	Day 0	Day 1	Day 7	Day 14
WBC ($4.36\text{--}11.90 \times 10^3/\mu\text{L}$)	8.75 ± 0.52	16.14 ± 4.22	9.63 ± 0.65	10.72 ± 0.88
RBC ($5.68\text{--}8.80 \times 10^6/\mu\text{L}$)	6.66 ± 0.42	6.01 ± 0.02	6.46 ± 0.21	6.44 ± 0.31
Platelets ($190\text{--}468 \times 10^3/\mu\text{L}$)	293.50 ± 15.50	291.50 ± 12.50	351.50 ± 6.50	272.00 ± 12.00
CRP (0.0–10.0 mg/L)	5.10 ± 0.20	$64.90 \pm 13.40^*$	7.35 ± 0.95	7.20 ± 1.40
Glucose (75–126 mg/dL)	89.50 ± 6.50	115.00 ± 9.00	102.50 ± 1.50	94.00 ± 14.00
SU (11–27 mg/dL)	14.50 ± 4.50	12.50 ± 2.50	14.00 ± 3.00	14.50 ± 2.50
Creatinine (0.5–1.4 mg/dL)	0.75 ± 0.15	0.65 ± 0.05	0.75 ± 0.05	0.80 ± 0.10
Albumin (3.2–4.3 g/dL)	3.50 ± 0.40	3.20 ± 0.40	3.35 ± 0.25	3.55 ± 0.35
Total bilirubin (< 0.2 mg/dL)	< 0.2	< 0.2	< 0.2	< 0.2
ALT (17–78 IU/L)	18.50 ± 2.50	14.00 ± 8.00	11.00 ± 0.00	15.00 ± 0.00
ALP (9–88 IU/L)	88.50 ± 5.50	129.00 ± 18.00	96.50 ± 10.50	95.50 ± 4.50
GGT (0–4 IU/L)	3.00 ± 0.00	3.00 ± 0.00	3.00 ± 0.00	3.00 ± 0.00
Sodium (145–152 mmol/L)	145.50 ± 1.50	147.00 ± 1.00	146.50 ± 0.50	146.00 ± 2.00
Potassium (3.6–5.3 mmol/L)	4.50 ± 0.30	4.40 ± 0.20	4.80 ± 0.20	4.70 ± 0.20
Chloride (107–115 mmol/L)	109.50 ± 0.50	111.50 ± 0.50	09.50 ± 0.50	108.50 ± 0.50
Bicarbonate (18–25 mmol/L)	22.50 ± 0.50	22.00 ± 1.00	22.00 ± 0.00	23.50 ± 1.50

Note: Statistically significant differences are identified with an asterisk. Reference ranges are provided in parentheses for each variable.

Abbreviations: ALT, alanine aminotransferase; ALP, alkaline phosphatase; CRP, C-reactive protein; GGT, gamma-glutamyl transferase; ICG, indocyanine green; RBC, red blood cells; SU, serum urea; WBC, white blood cells.

of ICG and no necrosis, inflammation or fibrosis was noted at either time point.

4 | DISCUSSION

Inhaled ICG provides rapid intraoperative NIRF imaging of normal pulmonary parenchyma and is clinically feasible. Dogs that received higher doses of ICG (0.5 and 1.0 mg/kg) had stronger fluorescence that persisted for at least 20 min when compared to the lowest dose evaluated in this study (0.1 mg/kg). Importantly, inhalation of ICG was not associated with any adverse clinical or histologic effects during or following administration, indicating a favorable safety profile for this novel technique.

Peak subjective and quantitative fluorescence occurred earlier in dogs that received 0.1 and 0.5 mg/kg of ICG and, paradoxically, later in dogs that received 1.0 mg/kg. One possible explanation for this observation is fluorescence quenching, a transient phenomenon in which high concentrations of fluorophores can lead to a reduction in observable fluorescence due to energy transfer interactions and absorption of light emitted by nearby ICG molecules.¹⁴ Alternatively, the delayed peak may be related to slower diffusion and distribution of ICG within the pulmonary parenchyma at higher doses, resulting in a more gradual increase in peak fluorescence. While all dogs included in this study were of similar bodyweight and maintained on consistent ventilator settings, it is also possible that subtle differences in minute ventilation may have impacted the timing of peak fluorescence. Future studies evaluating the pharmacokinetics of inhaled ICG and its distribution dynamics within lung tissue could provide further insights into this observation.

Fluorescence was sustained over the 20-min observation period in the higher dose groups (0.5 and 1.0 mg/kg) but diminished to baseline values within 16 min of administration in the lowest dose group (0.1 mg/kg). Additionally, both qualitative and quantitative fluorescence began to decline within our observation period in dogs that received 0.5 mg/kg of inhaled ICG, while remaining strong throughout the observation period in the 1.0 mg/kg group. These results are in contrast to a contemporary study in rabbits in which fluorescence at low doses (0.25 mg/kg) remained strong and persisted for over 24 h.⁸ These discrepancies may reflect variations in fluorescence sensitivity between NIRF imaging systems or inherent differences in physiology between species, with our results suggesting higher doses may be required for sustained fluorescence of normal pulmonary parenchyma in dogs compared to rabbits. Alternatively, it is possible that fluorescence may also persist in dogs 24 h after administration of inhaled ICG, particularly with

higher doses which demonstrated substantial fluorescence at the end of our observation period; however, the authors elected not to repeat thoracoscopy 24 h after ICG administration in this study due to concerns regarding animal welfare and lack of perceived added clinical value. Based on the findings of this study, the authors recommend utilizing the 0.5 or 1.0 mg/kg dose to facilitate strong and persistent fluorescence during thoracoscopic exploration. Our results suggest that a lower dose of inhaled ICG offers more immediate peak fluorescence, whereas higher doses provide stronger fluorescence with a later peak. These nuances may influence the dose chosen in a clinical setting when considering procedural goals and timing. Additional doses of inhaled ICG were not repeated during this study, therefore the effect of repeated dosing to enhance diminishing fluorescence remains unknown. Further refinement of inhaled ICG dose may be necessary based on the specific disease being evaluated.

Discrepancies between subjective and quantitative assessments of fluorescence may be attributed to several factors. Subjective evaluation by the surgeon could be influenced by ambient lighting conditions, visual adaptation to the NIRF imaging system, and variability in human perception of fluorescence. While more objective, quantitative fluorescence measurements are also affected by factors such as the angle and distance of the endoscope from the observed tissue, ROI selection, and potential interference from background fluorescence. Attempts were made to control for these factors by performing subjective intraoperative assessments in a dark operating room, use of a 0° endoscope, and positioning of the endoscope such that the medial aspect of the right cranial lung lobe occupied the field of view during peak inspiration to standardize the distance of the endoscope from the observed lung lobe at image capture. Postprocedural quantitative fluorescence was assessed using an ROI encompassing the entire surface of the imaged lung to minimize the influence of background fluorescence. Despite these efforts, there was some discrepancy between qualitative and quantitative assessment of fluorescence in this study which may reflect variability in human perception of fluorescence, observer bias influenced by the duration of administration of ICG, and subtle changes in distance between the pulmonary surface and endoscope during image acquisition; however, the authors contend that the settings of this study replicate the challenges that would be encountered in the clinical scenario and thus both subjective and quantitative fluorescence represent clinically useful data points.

Histologic evaluation revealed that ICG was localized primarily within the pulmonary vasculature rather than in the bronchioles or alveoli as expected. In rabbit and

mouse models with pulmonary tumors, Quan et al. reported that histologic distribution of inhaled ICG was isolated to healthy lung parenchyma and was not identified within tumor tissue. In that study, macrophages were also mainly distributed within healthy tissue, suggesting that inhaled ICG persisted within these regions due to macrophage uptake.⁸ No alveolar macrophages or other cells were identified in airspaces, and although expression of a macrophage marker was not assessed in this study, it is unlikely that macrophage uptake is responsible for persistent fluorescence in dogs. Notably, our findings suggest that inhaled ICG rapidly enters the pulmonary vasculature yet allows for persistent fluorescence. This explains the subjective and histologic finding of increased fluorescence intensity in areas of atelectasis, where the overlying alveoli are collapsed and underlying fluorescent signals contained within the vasculature are strongly visualized. Additionally, intravascular distribution of ICG within normal lung tissue raises the possibility that increased vascular permeability or altered clearance mechanisms may impact the distribution and appearance of fluorescence in disease states. Due to the high-volume and low-pressure nature of the pulmonary arterial system, pulmonary arterioles and venules have a similar appearance microscopically; therefore, the exact distribution of ICG within the vasculature could not be further characterized. Additional investigation of cellular and spatial distribution of ICG with specific pulmonary diseases is warranted to better understand the mechanisms driving fluorescence distribution and intensity following inhaled administration.

An elevation in CRP one day after surgery and inhalation of ICG is likely multifactorial. Surgical intervention is known to induce an acute-phase inflammatory response, reflected by increased CRP concentration. Additionally, general anesthesia and mechanical ventilation can contribute to transient systemic inflammation. Although no clinical signs or significant adverse events were observed, it is possible that the systemic inflammatory response was induced by exposure to ICG as foreign substances introduced into the respiratory tract can elicit localized or systemic reactions. The return of CRP to baseline by day 14 supports the hypothesis that this elevation was a temporary and expected response to the procedural interventions, though it is possible that inhaled ICG may have contributed to a systemic response. Additional evaluation with a control group would be necessary to further investigate this; however, given the absence of clinical signs and lack of deviations in monitoring parameters suggestive of respiratory or systemic insult, the authors do not feel that this is warranted.

The use of intraoperative NIRF imaging in combination with intravenous ICG has been adopted in several

human and veterinary procedures to improve patient safety and procedural success by highlighting anatomic structures of interest and delineating lesions.^{15–21} Intravenous ICG has shown promising initial results for bullae detection in people with primary spontaneous pneumothorax.^{22–24} In a canine model, intravenous administration of ICG resulted in the ability to identify emphysematous lesions that were not visible under white light using NIRF imaging; however, the average period of time during which fluorescence grossly differed between areas of abnormal and normal tissue diminished within 2 min.²⁴ While these results are promising, the brief time-frame reported for differentiation of lesions poses a challenge in aiding veterinary surgeons performing complete exploration of the thoracic cavity. Given the prolonged fluorescence demonstrated at higher doses explored in our study, inhaled ICG may offer improved detection of lesions in dogs with spontaneous pneumothorax.

The potential clinical utility of inhaled ICG to detect pulmonary lesions lies in its ability to generate sustained fluorescence of normal pulmonary parenchyma, which may provide contrast between areas of disease and healthy tissue in real time. According to Quan et al, inhalation of ICG in murine and rabbit lung tumor models resulted in distribution of fluorescence in normal pulmonary parenchyma with minimal to no fluorescence in tumor tissue, allowing for precise margin identification. In these models, the observed contrast in fluorescence between neoplastic and normal pulmonary tissue was due to mechanical airway obstruction caused by the tumor combined with alveolar uptake of ICG in healthy tissue.⁸ Distribution of inhaled ICG in dogs with spontaneous pneumothorax may also be altered by ruptured bullae, regional air leakage, and atelectasis, potentially resulting in heterogeneous fluorescence patterns. In these settings contrast between fluorescent, aerated lung and areas of diminished or absent fluorescence may aid in lesion localization. While the variables introduced by disease introduce complexity, they also underscore the importance of understanding fluorescence patterns in normal lung tissue. The findings of this study establish baseline fluorescence characteristics in normal lung tissue that may facilitate interpretation of altered fluorescence patterns in dogs with naturally occurring pulmonary disease.

Inherent limitations of this study are its exploratory nature and small sample size, limiting meaningful statistical comparisons of fluorescence between doses. The relatively short observation period also precludes the authors from determining the overall length of fluorescence provided by each dose; however, the results of this study demonstrate that this technique provides real-time clinically detectable fluorescence that persists for at least

the expected duration of exploratory thoracoscopy. Additionally, the subjective assessment of fluorescence by the operating surgeon, while blinded to dose, could also introduce observer bias. Given the pilot nature of this study and aim to evaluate the safety and clinical feasibility of inhaled ICG, it was elected not to administer ICG intravenously to any dogs; thus, comparisons of relative pulmonary fluorescence between routes of administration cannot be addressed by this study.

Importantly, this study utilized purpose-bred dogs with no underlying pulmonary disease. Fluorescence distribution, intensity, and persistence may differ in dogs with pulmonary pathology due to inflammation and alterations in airway architecture, vascular permeability, and ventilation and perfusion which may limit direct translation of these findings to clinical patients. Nevertheless, establishing safety and dosing parameters for NIRF of normal pulmonary tissue is a necessary prerequisite for responsible clinical translation of this novel technique, and the findings of this study provide foundational data to support further investigation of the effectiveness of inhaled ICG to identify lesions in dogs with naturally occurring pulmonary disease.

Analysis of fluorescence from a single lung lobe represents an additional limitation to this study. Because distance between the target tissue and near-infrared light source can dramatically affect fluorescence intensity, we sought to minimize the potential impact to subjective and quantitative fluorescence assessments by standardizing image acquisition for data analysis. The medial aspect of the right cranial lung lobe was chosen due to subjective ease of manipulation which allowed for rapid and consistent positioning, and a breath hold was also performed during each assessment to further minimize changes in distance caused by respiratory motion. While attempts were made to maintain camera positioning during 20 min of observation, all lung lobes were observed intermittently throughout the procedure and similar fluorescence was noted, therefore we believe the data obtained from the right cranial lung lobe is representative of diffuse pulmonary fluorescence. Nonetheless, it is possible that the anatomy of the bronchial tree may lead to relative differences in fluorophore distribution, resulting in differences in fluorescence intensity between lung lobes that were not identified in this study. This may be of particular interest when considering the caudal positioning of the accessory lung lobe.

In conclusion, inhaled ICG offers a promising new tool for thoracoscopic visualization of pulmonary parenchyma in dogs and the findings of this study support further investigation of this technique in clinical patients. Should inhaled ICG prove useful in the identification and

localization of pulmonary lesions in dogs with clinical disease, refinement of optimal doses for specific conditions will be warranted.

AUTHOR CONTRIBUTIONS

McPhetridge JB, DVM, DACVS (Small Animal): Contributed to study design, assisted in surgical case management, analyzed and interpreted data, drafted and revised the manuscript. Levine J, DVM: Contributed to study design, recorded data, and edited the manuscript. Oblak ML, DVM, DVSc, DACVS (Small Animal): Contributed to study design, interpreted data and provided in-line editing the manuscript. Lynch A, BVSc (Hons), DACVECC, MRCVS: Contributed to study design and provided in-line editing the manuscript. Foster RA, BVSc,³ PhD, MANCVS, DACVP: Contributed to study design, examined histologic sections and provided in-line editing of the manuscript. Scharf VF, DVM, DACVS (Small Animal): Contributed to study design, was responsible for surgical management of cases, oversaw data collection, interpreted data and provided in-line editing of the manuscript.

FUNDING INFORMATION

Funding was provided by the ACVS Surgeon-in-training grant and North Carolina State University Canine Research Endowment.

CONFLICT OF INTEREST STATEMENT

The authors have no conflicts of interest to disclose.

DATA AVAILABILITY STATEMENT

Data from this study was presented as an abstract in oral form at the American College of Veterinary Surgery Summit, Louisville, Kentucky, October 12, 2023.

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How to cite this article: McPhetridge JB, Oblak ML, Levine J, Lynch A, Foster RA, Scharf VF. Safety and feasibility of near-infrared fluorescence imaging with inhaled indocyanine green to assess the pulmonary parenchyma in six normal dogs. *Veterinary Surgery*. 2026;55(4):771-780. doi:10.1111/vsu.70104