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Clinical assessment of heartworm-infected Beagles treated with a combination of imidacloprid/moxidectin and doxycycline, or untreated

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Abstract

Background: Administration of moxidectin topically and doxycycline PO has been utilized experimentally as an alternative treatment for heartworm disease. However, clinical effects of this protocol remain poorly characterized.

Objective: To evaluate the clinical and postmortem findings associated with administration of doxycycline and monthly 10% imidacloprid + 2.5% moxidectin (IMD + MOX, Advantage Multi/Advocate) to *Dirofilaria immitis*-experimentally infected as compared to nontreated control dogs.

Animals: Sixteen purpose-bred, female, Beagle dogs.

Methods: Prospective, blinded, experimental study. Animals with surgically transplanted adult heartworms were randomized into 2 study groups of equal size: a nontreated control group (n = 8) and an IMD + MOX and doxycycline-treated group (n = 8). Randomization was performed using a complete block design according to circulating microfilarial concentrations, measured before treatment. Serum biochemical profiles, CBCs, thoracic radiographs and echocardiograms were performed prior to and 3 weeks after transplantation, and monthly for 10 months. Postmortem gross and histopathologic evaluations were performed.

Results: Compared to control animals, mean ± SD serum alanine aminotransferase (181 ± 203 U/L vs 33 ± 7 U/L; $P < .0001$) and alkaline phosphatase (246 ± 258 U/L vs 58 ± 19 U/L; $P < .0001$) activities were significantly higher in the treated group on day 28. Radiographic and echocardiographic evidence of heartworm disease was observed in both groups; however, no significant differences in these variables were noted between groups. Mean ± SD pulmonary arterial thrombus score was

Abbreviations: ALP, alkaline phosphatase; ALT, alanine aminotransferase; AT, pulmonary artery acceleration time; AT:ET, acceleration time index; ET, right ventricular ejection time; IMD + MOX, 10% imidacloprid + 2.5% moxidectin (Advantage Multi® for Dogs); L3, third-stage infective larvae; PA V_{max}, peak systolic pulmonary artery blood flow velocity; RPA, right branch pulmonary artery; RPAD, right pulmonary artery distensibility; TAPSE, tricuspid annular plane systolic excursion; TR V_{max}, peak systolic blood flow velocity of the tricuspid regurgitant jet.

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significantly higher in the treated vs nontreated group (3.9 ± 0.4 and 1.5 ± 2.1, respectively; P = .01).

Conclusions and Clinical Importance: The treatment protocol was well-tolerated with no clinically relevant adverse effects for any variable evaluated during the observational period.

KEYWORDS

clinical pathology, *Dirofilaria*, echocardiography, radiology

1 | INTRODUCTION

The causative agent of heartworm disease in dogs, *Dirofilaria immitis*, is a parasitic nematode containing the endosymbiotic bacterium, *Wolbachia*. Upon death of adult worms, the release of *Wolbachia* triggers a host inflammatory response by activation of Toll-like receptors.[1-3] Heartworm infection might lead to a syndrome that can include pulmonary lesions, *cor pulmonale*, and right-sided congestive heart failure. If untreated, complications including liver failure, renal failure, and death can occur.[4]

The American Heartworm Society (AHS) recommends administration of a 28-day course of doxycycline (10 mg/kg PO q12h), monthly preventive doses of a macrocyclic lactone, and a 3-dose regimen of melarsomine dihydrochloride for the treatment of heartworm infection in dogs.[5] While considered the clinical gold standard, use of this regimen might not be feasible during times of reduced melarsomine availability, for financially constrained dog owners, or in severely debilitated dogs. This has led veterinarians to seek an efficacious alternative adulticidal treatment option.

In recent years, several groups have evaluated the adulticidal efficacy of continuous monthly administration of prophylactic doses of macrocyclic lactones with and without coadministration of doxycycline.[1,6-9] Despite being generally effective for the eventual elimination of adult heartworms, these treatment protocols have longer time-to-effect than melarsomine-based regimens.[6-9] This prolonged period could allow for continued cardiopulmonary damage, supporting the AHS recommendation of a 3-dose melarsomine-based protocol.

Anecdotal reports from veterinarians, describing the conversion of heartworm antigen-positive dogs to antigen-negative status with the administration of monthly topical 10% imidacloprid + 2.5% moxidectin (IMD + MOX; Advantage Multi® for Dogs, Bayer HealthCare LLC, Shawnee Mission, Kansas) and doxycycline, led our group to evaluate the adulticidal efficacy of this drug combination in a controlled setting. In experimentally infected, heartworm-positive Beagles, IMD + MOX and doxycycline demonstrated 95.9% efficacy in eliminating sexually mature adult heartworms after 10 months of therapy and eliminated microfilariae within 21 days. While parasitological efficacy of this treatment regimen has been established,[10] descriptions of its clinical effects are limited.[11]

Arteriography, echocardiography, and radiography can be used to assess the severity of pulmonary vascular and parenchymal changes in heartworm disease and to evaluate for resolution of these changes after adulticidal treatment. Echocardiography also has utility for the noninvasive estimation of pulmonary artery pressure, relevant in dogs with heartworm disease that might develop pulmonary arterial hypertension. Using indices such as systolic time intervals,[12] peak tricuspid regurgitant flow velocity (TR V_{max}),[12] tricuspid annular plane systolic excursion (TAPSE),[13] and the more recently described right pulmonary artery distensibility (RPAD) index,[14,15] previous investigators have proposed cutoffs to categorize pulmonary arterial pressure and confirm or corroborate a diagnosis of pulmonary arterial hypertension.

The objective of this study was to describe the clinical and postmortem findings associated with doxycycline and monthly IMD + MOX, administered PO and topically, respectively, to dogs with experimental *D. immitis* infection compared to nontreated controls.

2 | MATERIALS AND METHODS

Sixteen female, purpose-bred Beagles, ranging from 17 to 34 months of age and weighing 9.2 to 14.5 kg, with no previous exposure to macrocyclic lactones, were used for this study. These dogs were experimentally infected by surgical transplantation of adult heartworms as part of a study designed to evaluate the adulticidal efficacy of IMD + MOX and doxycycline. Detailed study methods and data related to treatment efficacy have been published previously.[10] The Institutional Animal Care and Use Committee of the University of Georgia approved all activities (A2014 12-003-Y2-A5). This laboratory study was conducted in accordance with VICH GL9 Good Clinical Practices (GCP), June 2000 (FDA Guidance for Industry 85, May 2001) and applicable standard operating procedures.

After a 1-week acclimation period, approximately 30 minutes prior to anesthetic induction, dogs were premedicated with atropine (0.04 mg/kg IM), acepromazine (0.05 mg/kg IM), and butorphanol (0.4 mg/kg IM). Propofol (4.4 mg/kg IV, titrated to effect) was administered for the induction of anesthesia, the trachea was intubated, and inhaled isoflurane in 100% oxygen was administered for the maintenance of anesthesia. Dogs were positioned in right lateral recumbency and the left jugular furrow sterilely prepared for jugular venotomy. Eleven adult female and 5 adult male heartworms were surgically transplanted into the left jugular vein of each dog, as previously described.[10,16] All adult worms were obtained from dogs previously

TABLE 1 Schedule of study events

Study day(s)	Schedule of events
−37 to 282 ± 2	• Once daily general health observation
−34 ± 1	• Baseline (preinfection) radiographic and echocardiographic examinations, CBC, serum biochemical profile, quantification of circulating microfilariae, heartworm antigen testing
−28 ± 2	• Surgical transplantation of adult heartworms
−7 ± 2	• Quantification of circulating microfilariae • Randomization
−6 ± 1	• Baseline (postinfection) radiographic and echocardiographic examinations
0	• Baseline (postinfection) CBC, serum biochemical profile, quantification of circulating microfilariae, heartworm antigen testing • Administration of first dose of IMD + MOX and DOXY (treated group)
0-29	• Twice daily administration of DOXY (treated group)
1, 3, 7, 14, 21, 28	• Quantification of circulating microfilariae
22 ± 1, 50 ± 1, 78 ± 1, 106 ± 1, 134 ± 1, 162 ± 1, 190 ± 1, 218 ± 1, 246 ± 1, 274 ± 1	• Radiographic and echocardiographic examinations
28, 56, 84, 112, 140, 168, 196, 224, 252	• Administration of IMD + MOX (treated group) • CBC, serum biochemical profile, quantification of circulating microfilariae, heartworm antigen testing
280 ± 2	• Euthanasia for full necropsy

Note: IMD + MOX, Advantage Multi (moxidectin/imidacloprid) topical; DOXY, doxycycline, 10 mg/kg PO q12h.

experimentally-infected with infective third-stage *D. immitis* larvae (L3; Missouri 2015 [macrocyclic lactone susceptible] strain) and were harvested at 10 months after infection to ensure sexual maturity.[17] Isoflurane was discontinued after transplantation and dogs were allowed to recover.

Animals were randomized into 2 study groups of equal size, a nontreated control group (n = 8) and an IMD + MOX and doxycycline-treated group (n = 8). Because 1 of the aims of the original study was to evaluate microfilaricidal efficacy, randomization was performed using a complete block design according to circulating microfilarial concentrations prior to treatment, the latter obtained approximately 21 days after surgical transplantation of adult heartworms (day −7) by evaluation of thick smears and results of the modified Knott test, both performed using heparinized blood.

Treated dogs were administered IMD + MOX topically every 4 weeks for a total of 10 monthly treatments, and doxycycline (10 mg/kg q12h) for 30 days, with both treatments starting 4 weeks after transplantation (study day 0). Based on body weight measurements obtained on the day of each administration, either 1.0 mL or 2.5 mL IMD + MOX topical treatment was applied, according to the manufacturer's label recommendations. A placebo was not administered to dogs of the nontreated control group. Dogs were pair-housed in runs measuring 8′ × 16′ for the duration of the study and were not allowed regular on-leash or off-leash access to areas outside these runs.

Clinical data, including the results of radiography, echocardiography, CBC, and serum biochemical analysis, were collected for all dogs approximately 1 week before and 3 weeks after surgical transplant of adult worms, as well as every 4 weeks for the duration of the treatment period (Table 1).

All study dogs were monitored daily for the entire duration of this study as well as 1 hour after treatment with IMD + MOX and doxycycline. General attitude and behavior, capillary refill time, locomotion, appetite, and feces were recorded during these periods.

2.1 | Echocardiography

All echocardiographic examinations and measurements were performed by 1 of 2 board-certified veterinary cardiologists (AEC or GSR), each of whom was blinded to subject treatment group, utilizing an ultrasound unit (CX50, Philips Healthcare, Andover, Massachusetts) outfitted with a variable-frequency, 1 to 5 MHz, sector-array transducer. Dogs were sedated using 0.4 mg butorphanol/kg IV prior to each exam. Transthoracic 2-dimensional, M-mode, and spectral and color-flow Doppler images, with simultaneous electrocardiography, were recorded from standard right-sided and left-sided views,[18] with each animal manually restrained in lateral recumbency. Images were digitally stored for later analysis. Pulsed-wave spectral Doppler evaluation of pulmonary artery flow was performed from the right parasternal short-axis view, with a sampling gate placed centrally and at the level of the pulmonic valve. Color-flow and continuous-wave Doppler were used to identify tricuspid regurgitation and to record its velocity, when present. Parallel alignment of the Doppler beam and blood flow of interest was optimized by use of color-flow Doppler, and recordings from any imaging plane that produced an optimal lineup were used for final analysis. M-mode recordings of the proximal right pulmonary artery (RPA) were obtained from a right, parasternal, short-axis view that maximized RPA diameter, with the cursor aligned perpendicular to the long axis of the vessel. M-mode recordings of

the lateral tricuspid valve annulus were taken from a left, parasternal, long-axis view centered on the right ventricle, with the cursor aligned as parallel as possible to the right ventricular free wall.

2.2 | Echocardiographic assessments, measurements, and calculations

Subjective assessments of right ventricular size and wall thickness, right atrial size, and interventricular septal motion were made from standard 2D echocardiographic images and recorded for each study. The presence or absence of echocardiographic evidence of adult heartworms (ie, typical double-lined hyperechoic structures) in the right heart chambers and visible portions of the main and branch pulmonary arteries was recorded.[19] Degree of valvular insufficiency, if present, was subjectively graded as trace, mild, moderate, or severe, and this information was recorded.

For all M-mode and spectral Doppler-derived variables, the average of 3 measurements taken from consecutive sinus beats was used. Systolic time intervals were measured from pulmonary artery (PA) Doppler flow profiles as previously described. Pulmonary artery acceleration time (AT) was measured from the beginning of systolic PA flow signal to peak velocity.[12] Right ventricular ejection time (ET) was measured from the beginning to the end of the PA flow signal. Acceleration time index (AT:ET), was calculated as the ratio of acceleration time to ejection time. Peak systolic PA blood flow velocity (PA V_{max}) was recorded. Peak systolic blood flow velocity of the tricuspid regurgitant jet (TR V_{max}) was taken as the maximum measured velocity independent of imaging plane. TR V_{max} > 3.1 m/s was considered diagnostic for systolic pulmonary arterial hypertension, provided that PA V_{max} was normal (ie, < 2 m/s) and assuming a right atrial pressure of 6 mm Hg.[12] From M-mode recordings, TAPSE was taken as maximal longitudinal displacement distance, measured from the basilar-most to the apical-most position of the tricuspid valve annulus using a leading edge method, as previously described.[13] Maximum systolic and minimum diastolic internal diameters of the RPA (RPA_s and RPA_d, respectively) were measured from M-mode recordings using a trailing edge to leading edge method. RPAD index was calculated according to the following formula: RPAD index = $[(RPA_s - RPA_d)/RPA_s] \times 100$.[14,15]

2.3 | Radiographic studies

Four-view thoracic radiographic studies were obtained for each animal immediately after sedation with a single intravenous injection of 5 μg dexmedetomidine/kg body weight. Standard right and left lateral, ventrodorsal, and dorsoventral projections were obtained with a digital radiography system (MinXray HF80/15 + dlp), using exposure settings of 74 to 80 kV, 15 mA and 0.15 ms, and a focal distance of 101 cm. The effects of sedation were reversed with 50 μg atipamezole/kg body weight (ie, a volume equivalent to that of the previous dose of dexmedetomidine), administered intramuscularly immediately after completion of the radiographic study.

All radiographic studies were interpreted at a later date by a single board-certified radiologist (AS), who was blinded to dog identity, treatment group designation, and study day. The order of radiographic study interpretation was randomized across all time points and study subjects. Each radiographic study was evaluated using a consistent interpretive paradigm and anatomic checklist.

Radiographic variables were scored using a 6-point system to indicate lesion severity. According to this scoring system, radiographic variables for which findings were normal or the lesion/abnormality of interest was not detected were assigned a score of "0." If the particular lesion or abnormality *was* present, a score of 1 to 5 was assigned according to severity, where "1" = mild, "2" = mild-to-moderate, "3" = moderate, "4" = moderate-to-severe and "5" = severe. Radiographic variables evaluated in this manner included: bronchial pulmonary pattern, interstitial/alveolar pulmonary pattern, lobar pulmonary artery enlargement, lobar pulmonary artery tortuosity, lobar pulmonary artery blunting, main pulmonary artery enlargement, pleural effusion, pneumomediastinum, pneumothorax, pulmonary atelectasis, right-sided cardiomegaly, thoracic lymphadenomegaly, and vascular pulmonary pattern. For the purposes of this study, interstitial and alveolar pulmonary infiltrates were considered together, as these commonly represent a continuum of disease severity; interstitial patterns were assigned a score of 1 to 3 and alveolar patterns were assigned a score of 4 or 5. Lobar pulmonary artery enlargement was assessed using individual lobar vessels and not restricted to the principle caudal lobar arteries. Scores for a given radiographic variable were assigned by considering all 4 radiographic views. Instances in which no radiographic abnormalities were observed for the entire study were recorded. In addition to the radiographic variables presented above, the presence or absence of cardiac silhouette enlargement, pulmonary bullae, and abnormal pulmonary pattern of any type, were recorded.

2.4 | Postmortem and histopathologic analyses

Dogs were sedated intramuscularly with 0.4 mg/kg butorphanol and 0.02 mg/kg acepromazine prior to humane euthanasia using Beuthanasia-D solution (Intervet, Inc, Merck Animal Health, Madison, New Jersey), administered IV. The heart, lungs, and kidneys were removed, examined grossly, and samples were placed in 10% neutral-buffered formalin for fixation. The samples were processed routinely for histopathology. Hematoxylin and eosin-stained sections were analyzed by a board-certified veterinary pathologist (K. S.) who was blinded to treatment group.

Each histopathologic variable was scored using a unique scoring system (Table 2). Histopathologic variables for which findings were normal or the lesion/abnormality of interest was not detected were assigned a score of "0." For each dog and tissue, a single histologic section was examined.

TABLE 2 Scoring systems utilized for the evaluation of histopathologic variables in the present study

	Score				
Variable	**0**	**1**	**2**	**3**	**4**
Thrombus[a]	No histopathologic abnormalities	Vessel <25% occluded	Vessel 25%-49% occluded	Vessel 50%-74% occluded	Vessel >75% occluded
Thrombus[a]	No histopathologic abnormalities	Vessel <25% occluded	Vessel 25%-49% occluded	Vessel 50%-74% occluded	Vessel >75% occluded
Endarteritis	No histopathologic abnormalities	Leukocytes infiltration limited to intima	Up to 50% of vessel wall infiltrated	>50% of vessel wall infiltrated	Transmural infiltration
Intimal proliferation	No histopathologic abnormalities	Focal	Multifocal	Locally extensive	Diffuse
Perivascular cuffing	No histopathologic abnormalities	1 layer of leukocytes surrounding medium-caliber vessels	2-5 leukocyte layers	6-10 leukocyte layers	>10 leukocyte layers
Myxomatous degeneration	No histopathologic abnormalities	Focal	Multifocal	Locally extensive	Diffuse
Vascular proliferation	No histopathologic abnormalities	Focal	Multifocal	Locally extensive	Diffuse
Fibrosis	No histopathologic abnormalities	Focal	Multifocal	Locally extensive	Diffuse
Cardiac variables					
Right ventricular myocardial degeneration/necrosis	No histopathologic abnormalities	Rare, small, scattered foci	Multifocal, moderately-sized foci	Multifocal to coalescing foci	Locally extensive
Right ventricular myocyte hypertrophy	No histopathologic abnormalities	Mild	Moderate	Severe	
Tunica media hypertrophy	No histopathologic abnormalities	Mild thickening	Moderate thickening	Severe thickening	
Hepatic variable					
Glycogenosis	No histopathologic abnormalities	Multifocal and mild	Diffusely zonal	Diffuse and moderate	Diffuse and severe
Renal variable					
Interstitial fibrosis and glomerulosclerosis	No histopathologic abnormalities	Focal, mild fibrosis	Focal interstitial fibrosis and glomerulosclerosis	Locally extensive interstitial fibrosis and glomerulosclerosis (supportive of infarction)	

[a]Worst affected vessel in examined section considered.

2.5 | Statistical analyses

All analyses were performed using SAS V 9.3 (Cary, North Carolina) with the exception of the Conover tests, which were performed using R (R Core Team), package PMCMR V 4.1 (2016). There were 2 study baselines, 1 before transplantation (day −34 ± 1; "preimplantation baseline") and 1 after transplantation (day −6 ± 1 for echocardiographic and radiographic data; day 0 for CBC, serum biochemical data; "postimplantation baseline").

CBC, serum biochemistry, and echocardiographic data were analyzed using a linear mixed effects model. The full mixed model included fixed factors for group, study day, and a group by study day interaction and a random intercept for each dog. Multiple comparisons for CBC and serum biochemistry data were adjusted for using Tukey's test. For echocardiographic data, multiple comparisons between groups at each time point and between each time point and pretransplantation baseline (day −34 ± 1) and posttransplantation baseline (day −6 ± 1) were adjusted for using the Holm-Bonferroni method. Presence of adult heartworms in the proximal pulmonary artery segments was analyzed between groups at each time point using the Fisher's exact tests.

Radiographic data for the frequency of normal radiographs (instances including no radiographic lesions) and presence/absence of radiographic variable counts were compared between groups at each

study day using Fisher's exact test and between times for each group day using Cochran's Q test. Severity of radiographic variables was compared between groups at each study day using Mann-Whitney tests and between time points for each group using Friedman tests. The Hochberg procedure was used for adjustment of multiplicity. When the Friedman test was significant, multiple paired comparisons were performed using Conover's test with the Hochberg adjustment of multiplicity.

Histopathology scores were compared between groups by both Student's *t*-tests and Mann-Whitney tests. When only 2 scores were recorded, then Fisher's exact test was also used. Any *P*-value <.05 was considered statistically significant.

3 | RESULTS

All dogs completed the 10-month study period. The median (range) administered doxycycline dosage was 13.08 (10.0-14.1) mg/kg PO q12h. Assessment of treatment efficacy for this cohort has been described elsewhere in detail.[10] Briefly, and of relevance to the present study, all dogs tested positive for the presence of heartworm antigen on study day 0. Microfilariaemia was noted in all but 2 dogs (n = 1 from each treatment group) at study day −7 (randomization). No treated dog had detectable circulating microfilariae by 21 days after the first IMD + MOX administration. The mean number of adult heartworms recovered at necropsy was 10.6 and 0.6 in the nontreated control and treated groups, respectively.[10]

Two dogs developed transient (ie, <3 days in duration), self-limiting clinical signs and physical examination findings related to heartworm disease, including coughing (n = 1 nontreated control dog) and coughing, quiet demeanor, and auscultable pulmonary crackles (n = 1 treated dog), on study days 28 and 30, respectively. An additional dog in the treated group developed clinical signs (including lethargy, pale oral mucous membranes, and tachypnea), suspected to be related to acute pulmonary thromboembolism, on study day 173. These signs were self-limiting and resolved within 24 hours without medical intervention. No dog developed clinical signs consistent with caval syndrome.

3.1 | CBC and serum biochemistry findings

Pretransplantation and posttransplantation baseline hematologic variables were not different between treatment groups, and group means for these variables were within normal reference ranges both before and after heartworm transplantation. On study day 28, serum activity of alkaline phosphatase (ALP) was significantly higher in treated (246 ± 258 U/L) vs nontreated (58 ± 19 U/L) dogs (laboratory reference range, 11-131 U/L; $P < .0001$). Three treated, and no nontreated dogs had serum activity of ALP greater than the upper limit of the laboratory reference range, with values ranging from 2.6 to 5.3 times the upper limit of normal. Similarly, serum alanine aminotransferase (ALT) activity was significantly higher in treated (181 ± 203 U/L) vs nontreated (33 ± 7 U/L) dogs at study day 28 (laboratory reference range, 9-105 U/L; $P < .0001$) Three treated, and no nontreated dogs had serum ALT activities greater than the upper limit of the laboratory reference range, with values ranging from 1.6 to 5.7 times the upper limit of normal. No significant differences in ALT and ALP activities were noted between study groups at any other study timepoint. Within the treated group, ALP and ALT activities were significantly higher at day 28 than at all other tested time points ($P < .0001$ for all comparisons). No other clinically relevant, statistically significant differences in other CBC or serum biochemistry variables were noted between study groups at any time point or among study days within a treatment group.

3.2 | Radiographic findings

All radiographic studies were considered to be of adequate diagnostic quality. No radiographic abnormalities were detected in any dog prior to experimental infection. After surgical transplantation, various radiographic abnormalities were detected in dogs of both study groups. All dogs had at least 1 radiographic abnormality after infection, including the presence of an abnormal pulmonary pattern. However, there were no significant differences between study groups in the proportion of affected dogs or in the severity score for any evaluated variable at any time point.

Additional radiographic abnormalities noted at least once after transplantation in dogs of both study groups included mild pleural effusion (n = 2 dogs of each study group, all instances on study days ≤106 ± 1), pulmonary bullae (n = 4 nontreated, n = 3 treated), mild or mild-to-moderate right-sided cardiomegaly (n = 4 nontreated, n = 5 treated), mild thoracic lymph node enlargement (n = 2 nontreated, n = 3 treated), mild or mild-to-moderate main pulmonary artery enlargement (n = 8 nontreated, n = 6 treated), mild or mild-to-moderate lobar pulmonary artery enlargement (n = 7 in each study group), mild or mild-to-moderate lobar pulmonary artery tortuosity (n = 6 in each study group) and mild or mild-to-moderate lobar pulmonary artery blunting (n = 1 nontreated, n = 4 treated). An interstitial or alveolar pulmonary pattern was noted in all dogs of both study groups at least once after surgical transplantation. In both study groups, the severity score for this variable was significantly greater at the posttransplantation baseline (day −6 ± 1) compared to the pretransplantation baseline (day −34 ± 1); however, in the treated group, severity scores remained significantly higher than at the pretransplantation baseline for a total of 5/10 subsequent study days, whereas this was the case for only 1/10 subsequent study days in the nontreated control group (Figure 1).

Radiographic evidence of moderate-to-severe (n = 3) and severe (n = 1) pneumothorax with accompanying lung lobe atelectasis was observed in dogs of both study groups (n = 3 treated, n = 1 control) at the posttransplantation baseline (day −6 ± 1). In 2 dogs (1 from each study group), mild pneumothorax (without atelectasis)

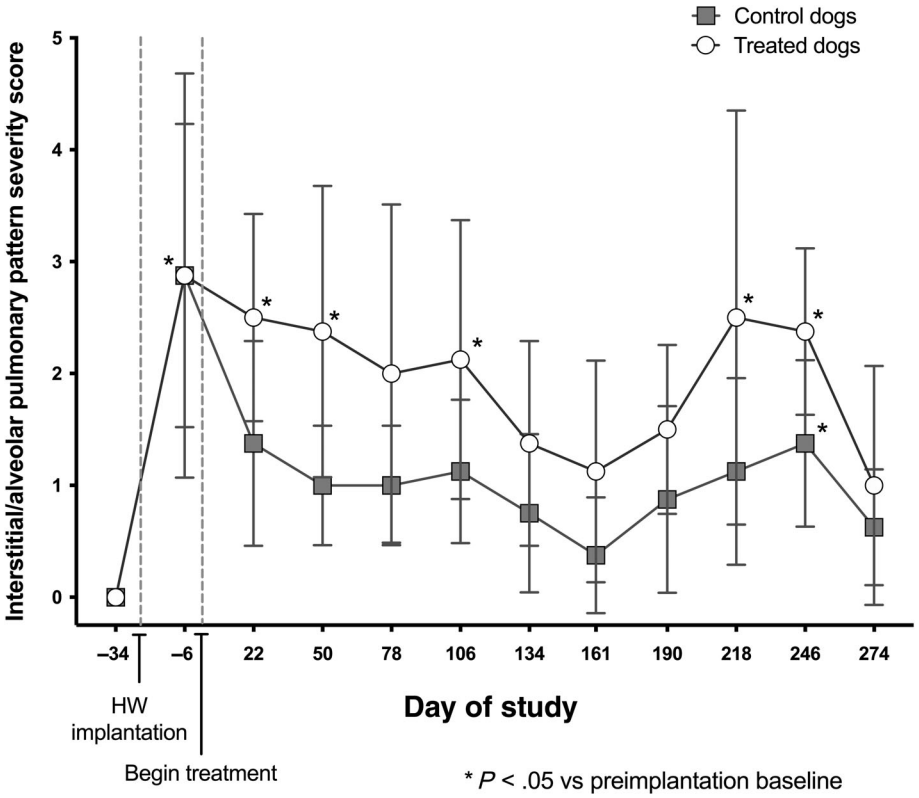

FIGURE 1 Mean ± SD radiographic pulmonary interstitial/alveolar pattern severity scores in *Dirofilaria immitis*-infected dogs treated (n = 8) or nontreated (n = 8) with doxycycline and monthly, topical, Advantage Multi® (10% imidacloprid +2.5% moxidectin; IMD + MOX)

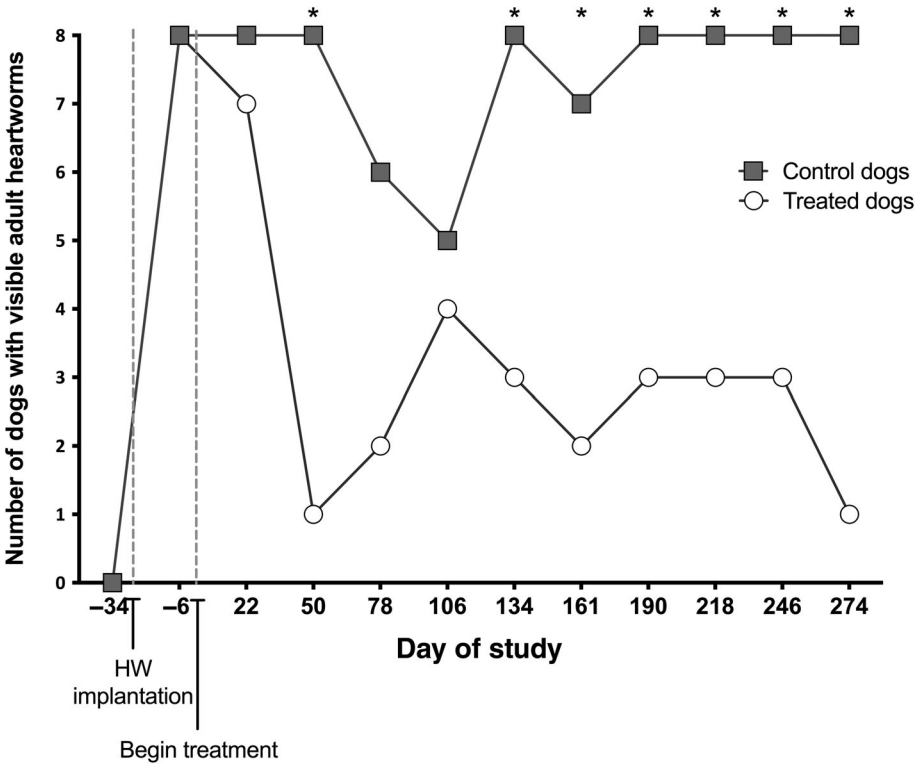

FIGURE 2 Frequency of *Dirofilaria immitis*-infected dogs treated (n = 8) or nontreated (n = 8) with doxycycline and monthly, topical, Advantage Multi® (10% imidacloprid +2.5% moxidectin; IMD + MOX), in which adult heartworms were detected by echocardiography in the proximal pulmonary artery segments. *, denotes significantly different ($P < .05$) proportion of dogs with this finding between treatment groups within given timepoint

persisted until study days 106 and 219, whereas this finding had resolved by day 22 ± 1 in the other 2 dogs. Concurrent pneumomediastinum or pulmonary bullae were not present in any of these studies. However, in 2 of these dogs (1 from each study group), pulmonary bullae were noted on at least 1 other study day when pneumothorax was not present.

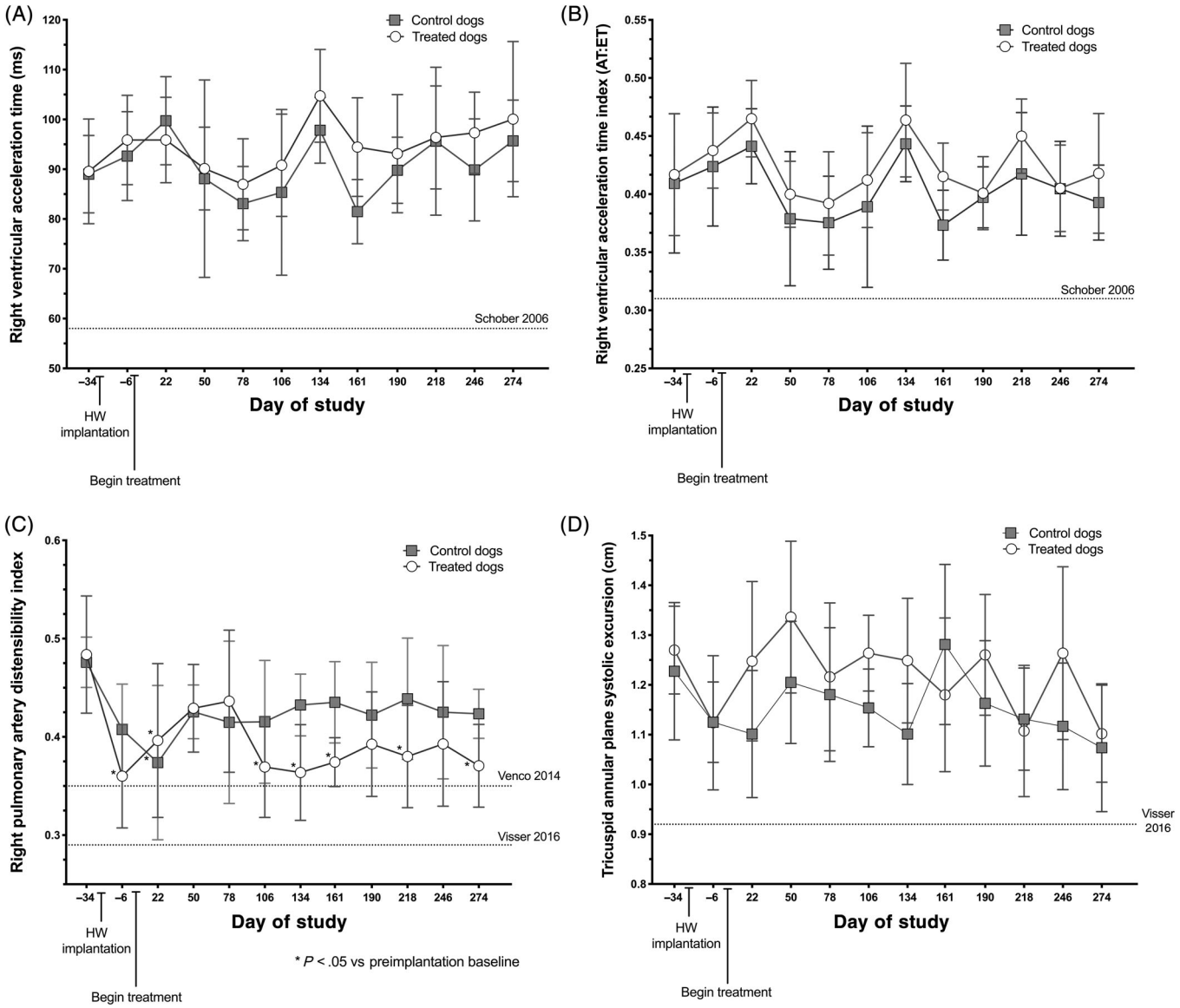

FIGURE 3 Mean ± SD echocardiographic right ventricular acceleration time (A), right ventricular acceleration time index (B), right pulmonary artery distensibility index (C), and tricuspid annular plane systolic excursion (D) in *Dirofilaria immitis*-infected dogs treated (n = 8) or nontreated (n = 8) with doxycycline and monthly, topical, Advantage Multi (10% imidacloprid + 2.5% moxidectin; IMD + MOX). Horizontal lines represent cutoffs, values below which suggest pulmonary arterial hypertension, advocated by several authors. (References: Schober and Baade,[12] Visser,[14] and Venco et al[15])

3.3 | Echocardiographic findings

Echocardiographic images of diagnostic quality were obtained in all dogs at all time points. Adult heartworms were visualized within the proximal portions of the pulmonary arteries in all dogs 3-weeks after transplantation (study day −6 ± 1). At each observed time point during the treatment period, fewer treated dogs had echocardiographically detectable worms compared to nontreated controls (Figure 2). During the treatment period, the median (IQR) number of dogs with visible heartworms at each study time point was 8 (6.75-8.00) and 3 (1.75-3.25) for nontreated controls and treated dogs, respectively. This difference was statistically significant between the treated and control groups on study days 50 ± 1, 134 ± 1, 162 ± 1, 190 ± 1, 218 ± 1, 246 ± 1, and 274 ± 1. Comparing the findings of the final echocardiographic study (study day 275) to those of necropsy, the calculated sensitivity and specificity of echocardiography to detect the presence of live adult heartworms or worm fragments was 82% and 100%, respectively. Both animals with false negative echocardiographic studies had low worm burdens; in 1, 2 live worms and 1 degenerate worm fragment were identified at necropsy, and in the other dog, a single, live, female worm was noted.

Subjectively, mild right ventricular enlargement was noted in 1 nontreated, control dog on 2 occasions (study days 162 ± 1 and 190 ± 1), and in 1 treated dog on 1 occasion (study day 246 ± 1). No

TABLE 3 Histopathologic scores from *Dirofilaria immitis*-infected dogs treated (n = 8) or nontreated (n = 8) with doxycycline and monthly, topical, Advantage Multi®P (10% imidacloprid + 2.5% moxidectin)

	Control (nontreated)	Treated	*P*-value
Pulmonary variables			
Thrombus	1.5 ± 2.1	3.9 ± 0.4	**.01**
Endarteritis	2.3 ± 1.6	2.5 ± 1.3	.74
Intimal proliferation	3.1 ± 1.0	2.4 ± 0.7	.11
Perivascular cuffing	2.6 ± 1.3	2.8 ± 1.3	.85
Fibrosis	1.8 ± 1.0	2.4 ± 0.7	.19
Myxomatous degeneration	1.4 ± 0.9	2.0 ± 1.1	.23
Vascular proliferation	1.6 ± 1.3	2.1 ± 1.5	.48
Pulmonary artery variables			
Endarteritis	0.9 ± 0.4	0.4 ± 0.5	.11
Intimal proliferation	1.9 ± 1.2	0.7 ± 1.0	.07
Myxomatous degeneration	2.1 ± 0.4	2.8 ± 1.0	.16
Fibrosis	2.1 ± 0.4	1.6 ± 0.1	.23
Cardiac variables			
Right ventricular myocardial degeneration/necrosis	0.9 ± 0.4	0.9 ± 0.4	1.0
Right ventricular myocyte hypertrophy	1.1 ± 0.6	1.1 ± 0.4	1.0
Tunica media hypertrophy	0.5 ± 0.9	0 ± 0	.17
Hepatic variable			
Glycogenosis	2.1 ± 1.0	1.9 ± 1.1	.64
Renal variable			
Interstitial fibrosis and glomerulosclerosis	0.5 ± 0.8	0.5 ± 0.8	1.0

Note: All results are presented as mean ± SD. Bold values indicate statistically significant values $P < 0.05$.

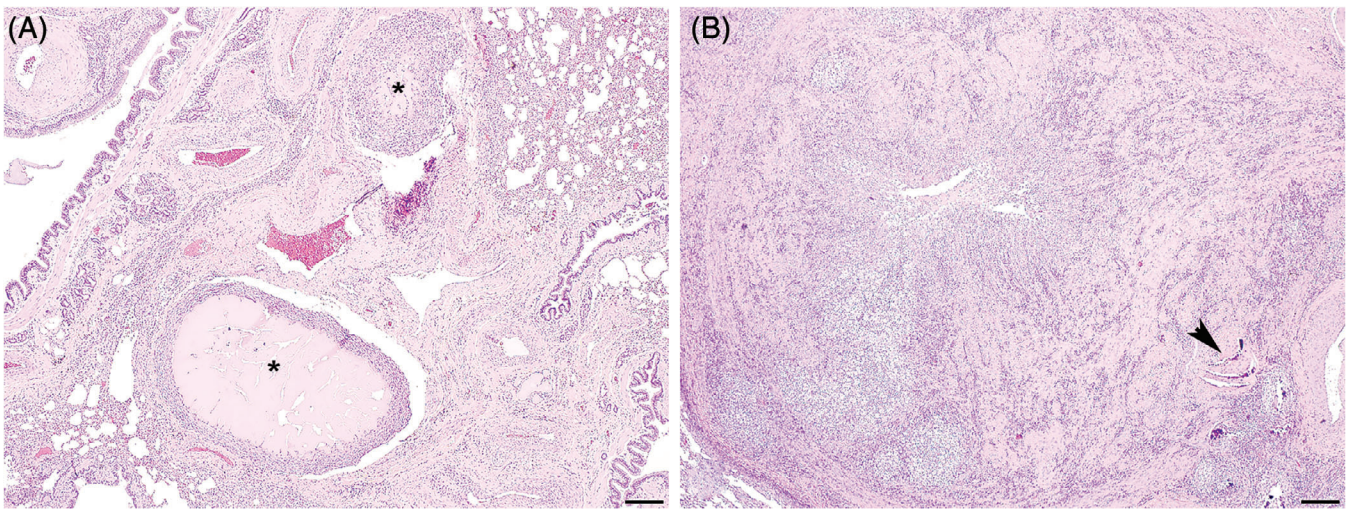

FIGURE 4 Photomicrographs of lung samples (40X magnification, bar = 200 μm) highlighting most severely affected pulmonary arteries recovered at necropsy for (A) nontreated and (B) treated dogs. A, Pulmonary artery branches contain degenerating worm remnants (asterisks) that nearly occlude vessels and are surrounded by leukocytes. B, The pulmonary artery branch is effaced by inflammatory cells and collagen deposition, with neovascularization (arrowhead)

dog developed echocardiographic evidence of right atrial or proximal pulmonary artery dilatation, right ventricular concentric hypertrophy, or abnormalities of interventricular septal motion.

Tricuspid regurgitation was present in 1 or more dogs at each time point, including pretransplantation baseline; in all cases, it was described as "trace" or "mild" in severity. Reliable measurements of TR

V_{max} were obtainable in only 4 dogs for a total of 9 instances; in each, measured TR V_{max} was not diagnostic for pulmonary arterial hypertension.

No statistically significant differences were noted in any evaluated echocardiographic variable between study groups at any time point. Echocardiographic indices of right ventricular systolic loading are presented in Figure 3. Compared to the pretransplantation baseline (study day −34 ± 1), RPAD index was numerically lower in both groups at all later time points; however, this reduction was statistically significant only for dogs treated with IMD + MOX and doxycycline (Figure 3C).

3.4 | Histopathologic findings

Of the histopathologic variables analyzed, only pulmonary arterial thrombus score was significantly different between treatment groups (3.9 ± 0.4 and 1.5 ± 2.1 in treated and nontreated dogs, respectively; P = .01; Table 3, Figure 4).

4 | DISCUSSION

In the present study, when compared to nontreated heartworm-positive control dogs, few differences in hematologic, radiographic, or echocardiographic variables were identified in dogs treated with IMD + MOX and doxycyline for a total of 10 months. Aside from significantly fewer treated dogs having echocardiographic evidence of adult heartworms at all study timepoints, any differences identified are considered of minimal or indeterminate clinical relevance. These data seem to indicate that the risk of clinically relevant complications in dogs treated with the tested protocol is comparable to that of nontreated dogs. With 1 exception (ie, pulmonary arterial thrombosis score), cardiopulmonary histopathologic changes were similar between study groups, suggesting that treatment with IMD + MOX and doxycycline was not associated with significant worsening or improvement in pulmonary pathological outcomes over the 10-month study period. The significantly higher pulmonary arterial thrombosis score noted in treated, as compared to nontreated, dogs is expected with adulticidal therapy, as worm die-off and embolism can worsen vascular damage acutely.[7] Although the long-term clinical relevance of this finding is unclear, the lack of concurrent detectable differences in echocardiographic indices of right-heart function and pulmonary hypertension, radiographic findings, and hematologic variables between study groups argues against its importance over a 10-month treatment period.

Several diagnostic imaging techniques have been used by others to assess the severity of pulmonary vascular and parenchymal changes in dogs with heartworm disease and to evaluate for resolution of lesions after adulticidal treatment. Serial pulmonary arteriograms were obtained in 7 Beagles infected with 50 *D. immitis* L3 before and after adulticidal treatment with thiacetarsamide. In that study, enlargement of the main pulmonary artery was observed as early as 6 months after infection, with the most severe enlargement observed 12 months after infection. Twelve months after adulticidal treatment and successful elimination of adult heartworms, dilatation of the caudal lobar pulmonary arteries was persistent, though a notable decrease in main pulmonary artery size was observed.[20]

Transthoracic Doppler echocardiography provides a noninvasive method to diagnose and estimate the severity of pulmonary arterial hypertension,[21] a sustained, pathological increase in pulmonary arterial pressure that can be caused by pulmonary arterial proliferative endarteritis and thrombosis in dogs with heartworm disease.[22,23] One such echocardiographic measurement, the RPAD index, has been validated against invasively determined RPA systolic blood pressure measurements in heartworm-infected dogs, and a cutoff value distinguishing normal from mild pulmonary hypertension has been proposed based on these data.[15] In naturally-infected heartworm-positive dogs, RPAD index has been used to evaluate changes in pulmonary hypertension presence and severity after adulticide therapy.[24] When compared to pretreatment baseline, no change in RPAD index was noted after treatment with a 3-dose melarsomine-based protocol in 34 client-owned dogs,[24] of which 23 (68%) had pulmonary hypertension at the time of diagnosis. However, dogs were evaluated relatively soon after adulticide therapy—approximately 60 days after the first, and 30 days after the second and third, melarsomine injections—suggesting that either more time after adult heartworm death might be necessary to observe expected changes in pulmonary artery pressures, or, less likely, that substantial reverse-remodeling of the pulmonary vasculature does not occur after elimination of adult worms in chronic infections. In the present study, with the exception of the RPAD index, echocardiographic indices of right ventricular systolic loading (expected to increase in chronic heartworm infection), were unable to demonstrate changes in systolic pulmonary artery pressure over time, or an effect of treatment. While experimental induction of heartworm disease by adult worm surgical transplantation provides infection uniformity that is useful for evaluation of treatment efficacy, it is likely that the disease induced by this model does not mirror natural infection perfectly. These "instant infections" likely require more time than that provided in the present study to induce the cardiopulmonary damage that is seen in long-standing, naturally occurring heartworm infections. Nonetheless, compared to the pretransplantation baseline (study day −34 ± 1), RPAD index was significantly lower (indicating higher pulmonary arterial systolic pressures) in treated, but not nontreated, dogs at multiple time points in the study, although treatment group means were not significantly different at any study time point and group means remained above 2 cutoffs[14,15] previously advocated as indicating the presence of at least mild pulmonary hypertension (Figure 3C). At study end, measured RPAD index was suggestive of mild pulmonary hypertension in n = 2 treated dogs and no nontreated dogs, whereas RPAD index was consistent with normal pulmonary arterial pressures in all dogs at preimplantation baseline. These data might suggest that treatment with IMD + MOX is associated with marginally higher pulmonary arterial pressures when compared to no treatment, although the clinical significance of this is currently unclear.

Longitudinal evaluation of the nontreated control group illustrates that this model was associated with radiographically detectable changes in the cardiopulmonary system, although most radiographic abnormalities were mild or mild-to-moderate in severity, with the exception of pneumothorax. Spontaneous pneumothorax has been described in dogs with naturally occurring heartworm disease, in which the cause was suspected to be pulmonary parenchymal infarction with subsequent bulla formation and rupture, or rupture of a secondary bacterial abscess.[25-28] One necropsy-based study of n = 15 naturally-infected heartworm-positive dogs, all dying because of acute, severe respiratory distress and collapse, found that pulmonary lesions leading to pneumothorax were confined almost exclusively to the dorsal, peripheral margin of the right caudal lung lobe, and were characterized as "pulmonary cavities" or scarring emphysema with perforation and formation of a bronchopleural fistula.[26] Radiographic evidence of spontaneous pneumothorax was noted in 4 of 16 dogs (25%) of the present study approximately 3 weeks after surgical transplantation of adult worms, and might have been because of thromboembolic complications leading to pulmonary infarction and airway rupture with or without bulla formation. Seven of 16 (48%) dogs in the present study had radiographic evidence of pulmonary bulla formation at some point in the 10-month period evaluated.

Compared to pretreatment baseline, radiographic interstitial/alveolar pattern score was significantly higher in treated dogs at 5 of 10 timepoints after initiation of therapy, while this was the case for only 1 of these 10 timepoints in nontreated dogs. The clinical relevance of this finding appears to be minimal, as only 2 dogs—1 of each group—displayed outward clinical signs (eg, cough) suggestive of pulmonary parenchymal lesions.

A previous study evaluating pathologic changes in dogs with heartworm disease, before and after adulticidal treatment, noted a clinically insignificant increase in circulating basophil concentration and decrease in circulating platelet concentration during infection, as well as a transient increase in monocytes, eosinophils, and ALP.[29,30] In the present study, serum ALP and ALT activities were significantly higher in treated, as compared to nontreated, dogs on study day 28 ± 1. Circulating activities of these enzymes were greater than the upper limit of the laboratory reference ranges in 3/8 (37.5%) treated dogs, with values ranging from 2.6 to 5.3 and 1.6 to 5.7 times the upper limit of normal for ALP and ALT, respectively. In all cases, these elevations were transient; ALT activities were within the normal laboratory reference range at the next scheduled recheck (ie, day 56 ± 1) in all 3 dogs, and ALP activities were normal by day 56 ± 1 in 1 dog and day 84 in the remaining 2 dogs. In 1 of these dogs, ALP was also mildly elevated (204 IU/L; laboratory reference range, 11-131 U/L) at preimplantation baseline (day −34 ± 1), making the significance of its day 28 elevation (346 U/L) unclear.[31]

Treated dogs within this study were paired-housed in 8′W × 16′L runs, with moderate exercise restriction. One treated dog developed clinical signs suspected to be associated with a pulmonary thromboembolism on study day 173, after 6 months of topical IMD + MOX. All adulticidal heartworm treatments carry a risk of pulmonary thromboembolism. While the risk of pulmonary thromboembolism can be lowered with exercise restriction, knowledge of the timeline for adult heartworm death after a treatment regimen is necessary to determine the appropriate duration of exercise restriction for that protocol. Since the timeline of adult heartworm death is unknown for the adulticidal treatment in this study, exercise restriction for the entire treatment timeline is the safest approach. Necessarily, use of the studied protocol would require a longer exercise restriction period than that currently recommended by the American Heartworm Society's 3-dose melarsomine treatment protocol.

There are limitations to this study. Parallel evaluation of a heartworm-infected group undergoing a melarsomine-based treatment protocol would have allowed for direct comparison of the tested protocol to current standard-of-care therapy. Additionally, dogs of the present study were infected with adult, as opposed to larval stage, heartworms. While this was done to standardize worm burden for evaluation of treatment efficacy, the relatively short period between infection and treatment initiation is unlikely to have produced disease that perfectly reflects that associated with natural infection.

As previously reported, IMD + MOX and doxycycline was successful in eliminating microfilariae in all dogs within 3 weeks and 95.9% efficacious at eliminating adult heartworms within 10 months.[10] The treatment protocol was well tolerated in these heartworm-positive dogs with no clinically relevant effects detected for any of the biochemical, pathological, or imaging variables evaluated during the 10-month observational period. The histopathologic cardiovascular and parenchymal pulmonary changes noted in dogs of this study are as expected for heartworm infection and any kind of adulticidal treatment. Even though the nature and time course of heartworm death after IMD + MOX and doxycycline treatment is unknown and might be slower than that of melarsomine, caution should still be exercised as pulmonary thromboembolism and other systemic effects are possible sequelae similar to other treatment protocols. Further work is needed to determine the long-term effects of this "slow kill" therapy in comparison to other slow kill regimens and melarsomine-based protocols.

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CONFLICT OF INTEREST DECLARATION

This study was funded by Bayer HealthCare Animal Health LLC. Cameon Ohmes and Joe Hostetler are employees of Bayer HealthCare Animal Health.

OFF-LABEL ANTIMICROBIAL DECLARATION

Doxycycline was administered at a dosage of 10 mg/kg PO q12h for 28 days.

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE (IACUC) OR OTHER APPROVAL DECLARATION

All data were generated and recorded at the University of Georgia in accordance with IACUC standards (Approval number: A2014 12-003).

HUMAN ETHICS APPROVAL DECLARATION

Authors declare human ethics approval was not needed for this study.

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