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Plasma nitric oxide in dogs with pulmonary hypertension secondary or not to left-sided heart disease

[Óxido nítrico plasmático em cães com hipertensão pulmonar secundária ou não à doença cardíaca esquerda]

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ABSTRACT

Nitric oxide (NO) is an important mediator responsible for vasodilation in pulmonary hypertension (PH) in humans. Based on human literature, it is suggested that in dogs there is also NO production decrease in lung tissue in the presence of PH with hypoxia. Therefore, the aim of this research was to determine the indirect plasmatic NO concentration in dogs with PH secondary or not to the left-side heart disease (LHD) and also with low, intermediate and high probability of PH to characterize the NO involvement on PH in dogs. Blood samples were collected from 35 dogs with probability of PH to NO measurement. NO concentration was estimated by the nitrite/nitrate concentration, and it was significantly different (p=0.002) in dogs with PH secondary to LHD (median=14 µM, range 11.19-16.59) and not secondary to LHD (median=25.88µM, range 15.08-36.71). However, this was not significant for the probability of low, intermediate, and high PH, although there was a tendency for NO concentration to be higher in dogs with high PH. The results of this study demonstrate that there is release of NO in dogs with PH, as well as that its dosage could differentiate dogs with PH secondary to LHD from dogs with non-secondary PH.

Keywords: hypoxia, nitrate, nitrite, pulmonary arterial pressure, pulmonary vasculature

RESUMO

O óxido nítrico (ON) é um importante vasodilatador na hipertensão pulmonar (HP) em seres humanos. Baseado na literatura humana, sugere-se que em cães também ocorra a diminuição da produção de ON no tecido pulmonar na presença de HP frente à hipóxia. Dessa forma, o objetivo desta pesquisa foi determinar a concentração indireta do ON plasmático em cães com HP secundária ou não à doença do lado esquerdo do coração (LHD), bem como caracterizar os achados ecocardiográficos de diagnóstico e a probabilidade de HP (baixa, intermediária e alta). Para isso, foram coletadas amostras de sangue de 35 cães com probabilidade de HP para mensuração de ON. A concentração do ON foi estimada pela concentração de nitrito/nitrato, e esta foi significativamente diferente (P=0,002) nos cães com HP secundária à LHD (mediana=14µM, intervalo 11,19-16,59) e não secundária à LHD (mediana=25,88µM, intervalo 15,08-36,71), porém não significativa para a probabilidade de HP baixa, intermediária e alta, embora houvesse uma tendência a ser maior a concentração de ON nos cães com probabilidade alta. Os resultados deste estudo demonstram que há liberação do ON em cães com HP, bem como que sua dosagem conseguiu diferenciar cães com HP secundária à LHD de cães com HP não secundária.

Palavras-chave: hipóxia, nitrato, nitrito, pressão arterial pulmonar, vasculatura pulmonar

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INTRODUCTION

Nitric oxide (NO) is a molecule discovered in 1980 as responsible for vascular relaxation induced by acetylcholine and at that time it became known as the endothelium-derived relaxing factor (Furchgott and Zawadzki, 1980; Palmer *et al*., 1987). Several cells synthesize NO from arginine through nitric oxide synthase (NOS) which is presented in three isoforms, two constitutive (cNOS) and one induced (iNOS) (Cerqueira and Yoshida, 2002). NO acts physiologically as a messenger in the main systems of the organism, a key determinant of endothelial function with endocrine, autocrine, and paracrine action in the heart, in addition to affecting the nervous and immune systems (Levine *et al*., 2012).

In blood vessels, NO plays a role in regulating the diameter of blood vessels and in vascular resistance by the ability to relax vascular smooth muscle (Levine *et al*., 2012). In addition, there is a relationship with arterial thrombosis when this gas is reduced (Cerqueira and Yoshida, 2002). When diluted, NO has a half-life of less than 10 seconds due to its rapid oxidation to nitrite and nitrate, therefore it is difficult to measure NO directly and to use such measurements to estimate the overall NO turnover in the body. NO binds to hemoglobin and other proteins that contain the heme nucleus leading to the end of its biological activity (Flora Filho and Zilberstein, 2000). Thus, NO production can be determined by measuring its metabolites in plasma, as well as the activity of endothelial NOS (eNOS) (Kleinbongard *et al*., 2003).

NO acts physiologically on the heart to adjust cardiac function through actions on inotropism, excitation-contraction coupling, diastolic function, heart rate and beta-adrenergic responsiveness. However, it is known that the activity of the eNOS and iNOS (induced) isoforms is altered during human heart failure (Cotton *et al*., 2002). The reduction of NO bioavailability is considered one of the common central factors in cardiovascular diseases in humans, although it is not clear whether this is the cause or the result of endothelial dysfunction (Naseem, 2005). In dogs, the treatment of endothelial dysfunction has been studied in pulmonary hypertension (PH), including cardiac patients (Jaffey *et al*., 2019). Dogs with myxomatous mitral valve disease (MMVD) and dilated cardiomyopathy demonstrated an increase in plasma nitrite and nitrate, but it did not correlate with medication use, heart disease severity or TNF-alfa and IL-1 activity (Laforcarde *et al*., 2003).

NO is also known to be involved in the pathophysiology of pulmonary arterial hypertension (PAH) in humans (Chester *et al*., 2017). In dogs, PH can develop mainly for three reasons: 1) secondary to pulmonary hyperflow, caused by congenital diseases with intra or extra cardiac defects with left-to-right shunt; 2) due to the increase in pulmonary vascular resistance (PVR), secondary to endothelial dysfunction derived from impairment in the pathway of prostacyclins and NO and also to diseases that cause lung parenchyma destruction; and 3) due to increased pulmonary venous pressure, secondary to diseases on the left side of the heart (LHD) (Reinero *et al*., 2020). Pulmonary hypertension is a multifactorial syndrome whose common result of all underlying causes is increased pulmonary vasculature resistance (Tabima *et al*., 2012). This resistance results from the vasoconstriction and the endothelial cell proliferation leading to obstructive pulmonary disease, which is mediated by the imbalance in homeostasis between vasoconstrictor (endothelin-1 and thromboxanes A2) and vasodilators (prostaglandin I2 and NO) agents, which favors vasoconstriction (Tabima *et al*., 2012).

The bioavailability of NO increases in inflammatory lung diseases, while it seems to be reduced in PAH in humans, which culminates in endothelial dysfunction, but, apparent controversies about the bioavailability of NO being normal or increased have also been reported, and they could be justified by methodological divergences, such as the selection of patients at different stages or severity of PH, or even by the higher activity of L-arginase II, an enzyme that competes with NOS and reduces the bioavailability of NO (Dias-Junior *et al*., 2008). On the other hand, there are no detailed studies that investigated the bioavailability of NO in dogs with PH, most studies involving NO in dogs with PH are related to the use of drugs as a source of NO for the treatment of PH, as phosphodiesterase type 5 inhibitors and inhaled nitrite (Cortés-Puch *et al.*,

2019, Akabane *et al*., 2020, Bueno *et al*., 2013; Brown *et al*., 2010; Jaffey *et al*., 2019, Hori *et al*., 2014). Given the importance of NO in the physiology of pulmonary circulation and involvement in the pathophysiology of PH, this molecule could represent a PH biomarker in dogs. Thus, the aim of this study was to determine the indirect plasma NO concentration in dogs with PH secondary or not to LHD, its stability over time (T1 and T2) and to point out the echocardiographic findings of diagnosis and characterization of the probability of PH.

MATERIALS AND METHODS

The prospective study evaluated dogs of clients with suspected PH. The domiciled animals were attended by the cardiology service of the Veterinary Hospital of São Paulo State University (UNESP), Jaboticabal campus, from January 2017 to December 2019 with suspected PH (ethics committee protocol nº 010488/18). The animals screened with suspected PH were evaluated and classified according to the probability of PH (Reinero *et al*., 2020) and it was identified whether the cause was secondary to LHD or not. Patients were diagnosed and treated according to the individual clinical need, as well as the returns that were scheduled. In the first evaluation, the guardians of dogs with suspected PH were invited to participate in the study and authorization was requested to collect a blood sample for the measurement of nitrite/nitrate before treatment (when it was needed) (T1). The owners of patients with suspected PH who attended the scheduled return (according to the needs of each patient) were again asked to consent to the collection of a second blood sample (T2) for the measurement of nitrite/nitrate.

Altogether, 72 animals were identified with suspected PH during the study period, which covered 20 months. Nitric oxide was estimated in 42 dogs that had PH and three were excluded because they did not meet the inclusion criteria. From the 42 animals with PH and with measured NO, 7 outliers were identified using the boxplot and were later excluded from this analysis. Thus, 35 animals with PH and whose NO were measured remained in the study. In a second step, from these 72 animals at the beginning of the study, 24 attended the return visit and the NO was dosed again in 11 animals.

As part of the inclusion criteria in the study, the animal should allow the physical examination, blood collection and complete echocardiographic examination to be performed. Once these steps were completed, the probability of pulmonary hypertension would be staged as low, intermediate, or high. Animals should have tricuspid insufficiency, measurable or not, and/or morphological changes in the right ventricle or pulmonary artery, as recommended by the PH consensus in dogs (Reinero *et al*., 2020) to determine the probability of PH. The echocardiographic signs that define the probability of PH include interventricular septum (IVS) flattening, left ventricular (LV) pseudohypertrophy, right ventricular free wall (RVFW) thickening (hypertrophy) or right ventricular (RV) dilation, pulmonary artery dilation assessed by the ratio with the aortic diameter (AP/Ao>1), pulmonary regurgitation velocity (PR) >2.5m/s, right pulmonary artery distensibility (RPAD) index <30%, pulmonary flow acceleration time (AT) <52-58 ms or low AT ratio in relation to the ejection time (ET) of the pulmonary flow (AT/ET <0.30) and notching in the deceleration of pulmonary flow.

The low probability of PH comprised dogs with tricuspid regurgitation velocity (TR) ≤3m/s or not measurable and with zero or one echocardiographic finding. The intermediate probability of PH comprised dogs with TR ≤3m/s or not measurable with two echocardiographic findings, TR between 3.0-3.4m/s with zero or one echocardiographic finding, or > 3.4m/s with no echocardiographic finding. The high probability of PH included dogs with TR ≤3m/s or not measurable with three echocardiographic findings, TR between 3.0-3.4m/s with ≥2 echocardiographic findings, or TR >3.4m/s with ≥1 echocardiographic finding.

The study exclusion criteria were animals that did not present echocardiographic findings suggestive of PH, when the guardian did not allow blood collection for NO measurement, and when the blood sample was of inadequate quality (eg hemolysis or lipemia). Animals that had RV outflow tract obstruction or congenital cardiac shunt were also excluded from the examination. Patients with PH and controlled concomitant diseases were not excluded, except if the

concomitant disease was in an acute stage or was the cause of clinical signs at the time of cardiological evaluation. Complete blood counts of the patients were performed, with the approval of the guardians, to check if there was an imbalance in the blood cells, such as anemia, polycythemia, leukocytosis, or leukopenia. Such abnormalities could be related to subclinical inflammatory factors related to the underlying cause of HP, therefore these changes did not fit the exclusion criteria. Patients who were already being treated for underlying diseases of PH were not excluded, since dogs with PH secondary to MMVD are usually stage C of congestive heart failure (CHF) (Keene *et al*., 2019).

Complete echocardiographic evaluation was performed on all animals with suspected PH to identify whether the underlying cause was LHD or not. Dogs were classified as PH secondary to LHD if they had left atrial remodeling, characterized by the left atrium/aorta (LA/Ao) ratio ≥1.6 (B2, C or D stage of CHF), according to the guidelines of MMVD (Keene *et al*., 2019) and not secondary to LHD with LA/Ao ratio ≤1.6 (A or B1 stage of CHF) Other variables evaluated were the left ventricular internal diameter in diastole (LVIDDN) and left ventricular internal diameter in systole (LVIDSN) normalized by weight, fractional shortening (FS), ejection fraction (EF), E wave (m/s), A wave (m/s), aortic flow velocity (m/s), mitral E' (m/s), mitral E/E', variables indicative of left cardiac remodeling and predictive of congestion.

Echocardiography was also used as a tool for the identification of right cardiac remodeling and systolic dysfunction, in addition to changes in the pulmonary artery for assigning the probability of PH according to the PH diagnostic and classification guidelines (Reinero *et al*., 2020). For this, the variables evaluated in this study were: TR (m/s), PA/Ao, AT/ET, right pulmonary artery distensibility (RPAD) index (%), tricuspid annular plane systolic excursion (TAPSE) indexed by weight ($mm/kg^{0.284}$), right ventricular internal length in diastole (RVILD) indexed by weight ($mm/kg^{0.33}$), right ventricular internal length in systole (RVILS) indexed by weight ($mm/kg^{0.33}$), apical right ventricular internal diameter in diastole (RVIDD_apical) indexed by weight ($mm/kg^{0.33}$), longitudinal right ventricular internal diameter in diastole (RVIDD_long) indexed by weight ($mm/kg^{0.33}$), right ventricular area in diastole (RVAD) indexed by weight ($cm^2/kg^{0.62}$), right atrial high indexed by weight ($mm/kg^{0.33}$), right atrial area (RAA) indexed by weight ($cm^2/kg^{0.71}$), right ventricular free wall in diastole and left ventricular free wall in diastole ratio (RVFWD/LVFWD), longitudinal right ventricular internal diameter in diastole and left ventricular internal diameter in diastole ratio (RVIDD/LVIDD).

NO was estimated using the indirect method of nitrate measurement from venous blood samples, as described by Stabile *et al*. (2016), collected in tubes containing 0.5 mL of heparin (10 U/mL) and kept in polystyrene boxes with ice until reaching the laboratory or stored in the refrigerator until the time of centrifugation. The samples were centrifuged in refrigerated centrifuges at 4ºC, 1200 g, for 20 minutes, and then the plasma was stored in microtubes and kept frozen at -80°C. For the measurement of nitrate, the samples were thawed and aliquots of 25.0µL of plasma were extracted, which were deproteinized by incubation with absolute ethanol at 4°C and kept for 30 minutes in a freezer (-20°C). Then, they were subjected to centrifugation (4000 g, 25ºC, 10 minutes) for later measurement. NO–ozone chemiluminescence method was used for the measurement of plasma nitrate using the Sievers® Nitric Oxide Analyzer 280 (GE Analytical Instruments, Boulder, CO, USA). Through this technique, there is a reaction that produces NO2 and emits an number of photons that is proportional to the concentration of NO (Ricciardolo *et al.*, 2004).

The results are expressed as the mean ± SEM or median and interquartile range. To test the normality of data, the Shapiro-wilk test was used, and for the homogeneity of variance, the Bartlett test was used.

For data with normal distribution, the ANOVA test and subsequent Tukey test were used. For data without normal distribution, the Kruskal-Wallis test and subsequent Dunn test were used to compare the echocardiographic variables and the value of NO in the PH not secondary to LHD and PH secondary to LHD and among the groups with low, intermediate and high PH probability.

To compare the NO value between time T1 and T2, the paired sample t-test or the Wilcoxon signed rank test was used.

Statistical analyses were considered significant at $p < 0.05$.

RESULTS

This study evaluated 35 dogs with probability of PH. The analysis of age, weight and sex were not significant for groups with PH secondary or not to LHD, while age was higher in dogs with intermediate probability of PH. The clinical variables are described in Table 1 and 2.

Table 1. Baseline data of clinical and echocardiographic variables of left heart side, right ventricle, and pulmonary artery from 35 dogs with pulmonary hypertension secondary or not to left heart disease

Clinical variables	PH not secondary to LHD	PH secondary to LHD	P value*
N cases	17	18	
Sex (male/female)	6/12	9/8	0.400
Age (years)	11 (10-13)	12 (11-13)	0.200
BW (kg)	7.4 (4.6-9.8)	8.6 (4.5-14)	0.488
NO (µM)	14 (11.19-16.59)	25.88 (15.08-36.71)	0.002

*P< 0.05. BW, body weight; NO, nitric oxide.

Table 2. Baseline data of clinical and echocardiographic variables of left heart side, right ventricle, and pulmonary artery from 35 dogs grouped based on probability of pulmonary hypertension

Clinical variables	Low probability	Intermediate probability	High probability	P value
N cases	12	9	14	
Sex (male/female)	5/7	4/5	6/8	1
Age (years)	13 (8.75-12) [b]	13 (12.75-15.25) [a]	12 (10.25-13) [ab]	0.011
BW (kg)	8.75 (5.10-10.55)	7.40 (4-10.8)	7 (4.78-14.33)	0.835
NO (µM)	13.51 (12.35-25.85)	15.91 (15.22-19.02)	18.94 (14.25-29.08)	0.380

Different letters indicate significant differences (P< 0.05) among groups. BW, body weight; NO, nitric oxide.

The results of complete blood counts of animals with NO measured were analyzed individually for the presence of anemia/polycythemia or leukocytosis/leukopenia. Not all patients had exams on the day of NO collection since it also depended on the tutor's authorization. 7/10 animals had a complete blood count on the day of diagnosis (T1), and only 2/7 had anemia. One of them was a female, Shih Tzu, 13 years old, with PH secondary to heart disease and high probability of PH (red blood cells 4,650,000/µL; NO = 14.27/µM) and which, in addition to anemia, also presented thrombocytosis, eosinopenia, lymphopenia and characteristics such as targeted red blood cells, mild anisocytosis, toxic neutrophils and atypical lymphocytes. The second animal with anemia (4,110,000/µL; NO = 73.78/µM) was a male, Poodle, 13 years old, with PH secondary to heart disease and high probability of PH. It also had mild leukocytosis (18,900/µL) due to neutrophilia, with the presence of mild anisocytosis and poikilocytosis. Still at T1, one female, Pinscher, 12 years old with low probability of PH not secondary to LHD, presented leukopenia (3,200/µL; NO = 14.72/µM) due to neutropenia and lymphopenia. At T2, 5/10 animals had blood count on the day of NO measurement, none of them had anemia, 2/5 had leukopenia, since one animal was the before mentioned female, Shih Tzu, 13 years old, with PH secondary to heart disease and high probability of PH (total leukocytes 5,800/µL; NO = 21.55/µM) due to lymphopenia and the other was a female, 13 years old Poodle (5,500/µL; NO = 12.55/µM) also due to lymphopenia.

Variables for identifying PH secondary to LHD and not secondary to LHD. The echocardiographic parameters LVIDDN, LVIDSN, LA/Ao, E wave, A wave, aortic flow velocity, mitral E', mitral E/E', related to left heart function and morphology, were efficient in differentiating patients with PH secondary to LHD and not secondary to LHD. The variables FS and EF presented no statistical difference. The data are shown in Table 3. Regarding the identification of dogs with low, intermediate, and high probability of PH, the echocardiographic parameters LVIDDN, LVIDSN, LA/Ao, E wave, A wave, aortic flow velocity, mitral E', mitral E/E', FS and EF were not significant (Table 4).

Plasma nitrate was able to differentiate dogs with PH secondary to LHD from dogs with PH not secondary to LHD (Table 1); however, it was not significant to differentiate the low, intermediate, and high probability of PH in dogs (Table 2).

Table 3. Baseline data of echocardiographic variables of left heart side, right ventricle, and pulmonary artery from 35 dogs with pulmonary hypertension secondary or no to left heart disease

	PH not secondary to LHD	PH secondary to LHD	P value*
Echocardiographic left heart variables			
LVIDDN	1.6(1.33-1.53)	1.82(1.6-2.17)	0.003
LVIDSN	0.77(0.68-0.85)	0.9(0.73-1.14)	0.033
EF (%)	78.62±7.42	78.99±7.36	0.886
FS (%)	46.24±8.02	47.17±6.93	0.717
LA/Ao ratio	1.51(1.29-1.66)	2.16(1.75-2.42)	0.000
E (m/s)	0.7±0.19	1.23±0.3	0.000
A (m/s)	0.77±0.28	1.09±0.24	0.002
E/A	0.755(0.755-1.105)	1.08(0.805-1.505)	0.171
Aortic flow velocity (m/s)	1.08±0.26	1.31±0.26	0.021
S' mitral (m/s)	0.94±0.32	1.1±0.32	0.171
E' mitral (m/s)	0.62±0.17	0.95±0.27	0.000
E/E' mitral	1.09(1.07-1.22)	1.41(1.15-1.72)	0.050
Echocardiographic right ventricle and pulmonary artery variables			
TR (m/s)	2.99(0.46)	2.89(0.68)	0.642
PA/Ao ratio	0.92(0.82-0.99)	1.04(0.94-1.17)	0.027
AT/ET (pulm flow)	0.45(0.12)	0.36(0.11)	0.031
RPAD Index	34.14(9.93)	30.46(12.36)	0.344
TAPSE ($mm/kg^{0.284}$)	6.93(2.13)	6.74(1.74)	0.782
$RVILD/kg^{0.33}$	11.31(2.63)	13.03(2.45)	0.054
$RVILS/kg^{0.33}$	8.58(2.02)	11.19(2.38)	0.001
$RVIDD\_apical/kg^{0.33}$	8.07(2.2)	8.62(2.67)	0.504
$RVIDD\_long/kg^{0.33}$	5.01(4.28-5.97)	4.78(3.68-6.93)	0.895
RVAD ($cm^2/kg^{0.62}$)	0.72(0.63-1.02)	0.93(0.65-1.03)	0.448
$RA\ high/kg^{0.33}$	9.5(1.81)	10.57(2.6)	0.165
RAA ($cm^2/kg^{0.71}$)	0.66(0.57-0.81)	0.95(0.57-1.10)	0.269
RVFWD/LVFWD	0.77(0.18)	0.85(0.16)	0.155
RVIDD/LVIDD_long	0.44(0.34-0.52)	0.3(0.24-0.38)	0.009

*P< 0.05. TR, tricuspid regurgitation; PA, pulmonary artery; Ao, aorta; AT, acceleration time; ET, ejection time; RPAD index, right pulmonary artery distensibility index; TAPSE, tricuspid annular plane systolic excursion; RVILD, right ventricle internal length in diastole; RVILS, right ventricle internal length in systole; RVIDD, right ventricle internal length in diastole; RVAD, right ventricle area in diastole; RA, right atrium; RAA, right atrium area; RVFWD, right ventricle free wall in diastole; LVFWD, left ventricle free wall in diastole; RVIDD_long, longitudinal right ventricle internal diameter in diastole; LVIDD_long, longitudinal left ventricle internal diameter in diastole.

Table 4. Baseline data of echocardiographic variables of left heart side, right ventricle, and pulmonary artery from 35 dogs grouped based on probability of pulmonary hypertension

Echocardiographic left heart variables				
	Low probability	Intermediate probability	High probability	P value
LVIDDN	1.57(0.29)	1.7(0.48)	1.7(0.41)	0.647
LVIDSN	0.84(0.77-0.9)	0.85(0.69-1.12)	0.74(0.65-1.03)	0.930
EF (%)	78.6(76.38-80.21)	77.14(72.50-83.17)	80.27(71.85-86.96)	0.876
FS (%)	45.78(4.53)	45.42(7.3)	48.3(9.43)	0.588
LA/Ao ratio	1.66(0.43)	1.86(0.46)	1.98(0.53)	0.293
E (m/s)	0.82(0.35)	1.06(0.31)	1.11(0.53)	0.255
A (m/s)	0.86(0.35)	1.05(0.31)	0.92(0.26)	0.401
E/A	0.92(0.8-0.9)	0.77(0.72-0.9)	0.93(0.79-1.45)	0.269
Aortic flow velocity (m/s)	1.13(0.36)	1.26(0.14)	1.22(0.25)	0.641
S' mitral (m/s)	0.9(0.34)	1.08(0.27)	1.15(0.23)	0.132
E' mitral (m/s)	0.6(0.18)	0.79(0.13)	0.74(0.18)	0.063
E/E' mitral	1.22(1.07-1.54)	1.14(1.09-1.38)	1.26(1.1-1.68)	0.719
Echocardiographic right ventricle and pulmonary artery variables				
TR (m/s)	2.51(0.33)^{b}	2.93(0.48)^{ab}	3.27(0.58)^{a}	0.004
PA/Ao ratio	0.95(0.83-1)	0.93(0.87-1)	1.07(0.93-1.22)	0.145
AT/ET (pulm flow)	0.43(0.1)^{a}	0.47(0.12)^{ab}	0.34(0.11)^{b}	0.023
RPAD Index	36.95(9.08)	33.02(11.38)	28.16(11.64)	0.130
TAPSE (mm/kg$^{0.284}$)	6.28(1.9)	7.02(1.89)	7.2(2)	0.465
RVILD/kg$^{0.33}$	12.62(2.49)	11.12(2.56)	12.72(2.75)	0.263
RVILS/kg$^{0.33}$	10.05(1.96)	8.77(2.32)	10.63(2.87)	0.172
RVIDD_apical/kg$^{0.33}$	6.68(1.84)^{b}	8.39(2.41)^{ab}	9.36(2.29)^{a}	0.029
RVIDD_long/kg$^{0.33}$	4.42(1.33)^{b}	4.28(1.14)^{b}	6.35(1.74)^{a}	0.002
RVAD (cm^{2}/kg$^{0.62}$)	0.72(0.63-0.94)	0.7(0.64-1.00)	1.03(0.67-1.47)	0.168
RA high/kg$^{0.33}$	9.41(1.73)^{ab}	8.8(1.67)^{b}	11.45(2.3)^{a}	0.005
RAA (cm^{2}/kg$^{0.71}$)	0.62(0.52-0.69)^{b}	0.58(0.51-1.10)^{ab}	0.97(0.74-1.27)^{a}	0.025
RVFWD/LVFWD	0.7(0.16)^{b}	0.86(0.17)^{ab}	0.87(0.15)^{a}	0.025
RVIDD/LVIDD_long	0.35(0.15)^{ab}	0.24(0.11)^{b}	0.5(0.12)^{a}	0.024

Different letters indicate significant differences (P< 0.05) among groups. TR, tricuspid regurgitation; PA, pulmonary artery; Ao, aorta; AT, acceleration time; ET, ejection time; RPAD index, right pulmonary artery distensibility index; TAPSE, tricuspid annular plane systolic excursion; RVILD, right ventricle internal length in diastole; RVILS, right ventricle internal length in systole; RVIDD, right ventricle internal length in diastole; RVAD, right ventricle area in diastole; RA, right atrium; RAA, right atrium area; RVFWD, right ventricle free wall in diastole; LVFWD, left ventricle free wall in diastole; RVIDD_long, longitudinal right ventricle internal diameter in diastole; LVIDD_long, longitudinal left ventricle internal diameter in diastole.

The time of the first evaluation and the second evaluation was variable among the animals because they obeyed a return deemed necessary according to the clinical need of each patient, with a minimum time of 28 days in symptomatic patients and a maximum of 308 days in asymptomatic (for example, CHF stage B2 dogs), with an average of 102.2 days for the 11 dogs that returned and had their NO measured in the second time to evaluate his stability. The statistical test showed that there was no significant difference in the NO in time 1 compared to time 2 (Fig. 1).

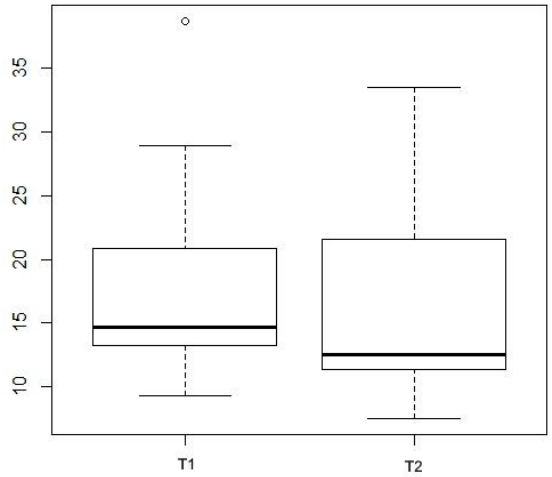

Figure 1. Plasma nitric oxide concentration of 11 dogs with probability of pulmonary hypertension on first (T1) and second (T2) day of collection.

The variables related to the right side of the heart and the pulmonary artery that were significant to differentiate PH secondary to LHD from PH non-secondary to LHD are: AT/ET, PA/Ao, indexed RVILS and RVIDD/LVIDD ratio. The other variables, such as TR, RPAD index, TAPSE, indexed RVILD (p=0.0504), indexed RVIDD_apical, indexed RVIDD_long, RVAD, RV high, RAA and RVFWD/LVFWD ratio were not significant (Table 3). However, when the analysis was performed considering the probability of PH, the variables AT/ET, RV high, RAA, TR, indexed RVIDD_apical, indexed RVIDD_long and RVFWD/LVFWD were statistically different (Table 4).

DISCUSSION

The nitrite anion is a cell signaling molecule considered a product of NO formation from NOS in the classical pathway. It may also be a NO-independent signal via the nitrate-nitrite-NO pathway, as nitrite is able to increase levels of cyclic guanosine 3′,5′-monophosphate (cGMP) and contribute to vasodilation in environments that are acidic and hypoxic, such as what occurs in the myocardium in the face of infarction (Liu *et al*., 2020). Previous studies had already demonstrated that the concentration of nitrite and nitrate appeared to be increased in both humans and dogs with heart disease (Winlaw *et al*., 1994; de Laforcade *et al*., 2003), a finding that was also observed in the results of this present study in which the NO concentration was significantly higher in dogs with pulmonary hypertension secondary to LHD. In fact, the NO concentration was even higher than the values demonstrated in research that determined baseline NO values in healthy dogs (median=0.00µM, range 0.00-6.16µM) compared to dogs with spontaneous heart disease (median=4.57µM, range 0.00-31.05µM, p=0.04) (de Laforcade *et al*., 2003). Similarly, the endothelium-derived relaxing factor (NO) by stimulation of alpha2-adrenergic receptors appears to be selectively increased in the coronary arteries of dogs with experimental CHF (O'Murchu *et al*., 1994). On the other hand, this was not observed in muscular arteries, and the authors suspected it occurred probably because the endothelial vasodilator function represents an important homeostatic mechanism, which in the face of a drop in cardiac output, selectively contributes to the preservation of coronary blood flow (O'Murchu *et al*., 1994).

Dogs with heart disease and left cardiac remodeling and alterations suggestive of PH evaluated in this study had a higher NO concentration than dogs with PH not secondary to LHD, although both values were above values already categorized for healthy dogs and dogs with heart disease (de Laforcade *et al*., 2003). The higher values found in the animals of our study could be explained by the probability of presence of PH that was estimated echocardiographically, as recommended by the guidelines. This differential factor in our study may have contributed to the finding of increased NO concentration in the groups evaluated. This fact probably implied in the absence of a significant difference in NO concentration when compared among the PH probability categories, although the results show a tendency for a higher NO concentration in dogs with a high PH probability.

An important point to be considered in our results is how NO formation occurred in the patients studied. NO bioavailability is influenced by the attenuation of NOS activity when there is acidosis in the ischemic tissue (Liu *at al*., 2020). We did not measure NOS, but due to the known inflammatory character of the underlying diseases, both MMVD and respiratory diseases could have a potential to induce iNOS activity (Belder *et al*., 1993). It has already been demonstrated in chronic CHF in humans that there is a negative regulation of eNOS, which decreases the bioavailability of NO and justifies changes in the endothelium, since eNOS is mainly expressed in the blood vessels endothelial cells (Le Corvoisier *et al*., 2000; Dias-Junior *et al*., 2008). Furthermore, cytokines stimulate iNOS, an enzyme that is also found in alveolar macrophages and has expression regulated by inflammatory mediators, that is, it is pro-inflammatory (Belder *et al*., 1993; Macdonald *et al*., 1996). This means that the increase in iNOS expression increases NO production, but in this situation, the excess of NO limits the inotropic capacity and exerts pro-apoptotic effects (Le Corvoisier *et al*., 2000).

However, this direct correlation between inflammatory substances such as TNF and IL-1 with NO has not been confirmed in dogs by de Laforcade *et al*. (2003). On the other hand, Fahim *et al*. (2004) demonstrated that cardiac remodeling can increase nitrite, nitrate, and TNF alpha concentrations in dogs with dilated cardiomyopathy. Fahim *et al*. (2004) observed that the concentration of nitrite and nitrate increases not only with the severity of heart failure, but also with the worsening of the left ventricular function, this was demonstrated by a decrease in ejection fraction, and an increase in left ventricular end-systolic and diastolic diameters. Therefore, cardiac remodeling may be a factor that increases the concentration of nitrite/nitrate, as seen in our result of dogs with PH secondary to left heart disease, since it was about elderly patients with chronic heart disease that led to the remodeling of the left heart to the point of culminating in suspected PH.

Since MMVD has been associated with endothelial dysfunction, Reimann *et al*. (2014) studied biopterin (BH4 and BH2) in dogs, an important cofactor for NO production, whose plasma concentration may reflect endothelial dysfunction and oxidative stress. They observed that biopterin was higher in the groups with symptomatic CHF (ACVIM class C) compared to the groups without symptoms (control, ACVIM B1 and B2); therefore, the authors concluded that the cardiac status is associated with the concentration of biopterin (Reimann *et al*., 2014). Although this study is not the same of measure nitrite/nitrate and also did not clarify whether the dogs had PH, the findings could support our study because of the similarity to characterize a possible endothelial dysfunction, since our dogs with PH secondary to MMVD also presented increased values of nitrite/nitrate and it is related to CHF as seen by de Laforcade *et al*. (2003) and Fahim *et al*. (2004).

Different results were found by Pedersen *et al*. (2003), that measured plasma nitrite and nitrate in dogs with and without mitral regurgitation and concluded that endothelial dysfunction occurs early in the development of mitral regurgitation in dogs (Cavalier King Charles Spaniels), and it was demonstrated as nitrite and nitrate concentrations decreased in animals with moderate to severe mitral regurgitation and no clinical signs (median 11.2 microM; interquartile range 6.9-17.1 microM; P = 0.02) compared to control group (median 20.0 microM; interquartile range 15.1-25.5 microM). Analyzing this, it should be considered that the LA/Ao ratio was not a classification criterion in the study by Pederson et. al (2003) and that dogs had an

LA/Ao ratio ≤1.6. In contrast, our study considered the LA/Ao ratio above 1.6 as a criterion to classify dogs with postcapillary PH and not the filling of the regurgitation in relation to the LA. That is, left cardiac remodeling and PH should be present in these dogs. Maybe, to MMVD, initially there may be endothelial dysfunction with a reduction in NO by eNOS (Le Corvoisier *et al*., 2000; Dias-Junior *et al*., 2008) and subsequently, by the activation of inflammatory factors and iNOS activity, NO increases (Belder *et al*., 1993). This could explain why our results presented higher nitrite and nitrate values in dogs with PH secondary to MMVD and lower in dogs with PH not secondary to MMVD, since in this last group the left cardiac remodeling was not present.

Hypoxia strongly inhibits the classical L-arginine pathway for NO production through activation of guanylate cyclase due to molecular O2 dependence. On the other hand, hypoxia stimulates the Nitrate-Nitrite-NO pathway that is largely independent of NOS, as is the case with muscle in exercise (Chirinos and Zamani, 2016). Moreover, enzymatic NO formation, in low oxygen and acidic conditions, from supplementing nitrite has been proposed for a wide variety of metal-containing enzymes and proteins, such as the heme-associated globins, mitochondrial proteins, molybdenum metalloenzymes, and NOS enzymes (Liu *et al*., 2020). Although there is different information regarding the down-regulation and up-regulation of eNOS in lung tissue, there is a consensus that NO signaling is impaired in the lungs of humans with PH and the reduction in its bioavailability may be related to impaired production or increased consumption (Tabima *et al*., 2012; Giaid and Saleh, 1995; Xue and Johns, 1995). These findings were not observed in our study due to two possible explanations, primarily due to involving patients with PH secondary to some underlying disease, and not just dogs with primary pulmonary hypertension as it is very common in human being. Another possibility is because the formation of NO in the lung tissue itself has not been evaluated, but it has been evaluated in peripheral blood. Unfortunately, we were not able to confirm diagnosis of underlying diseases of dogs with PH not secondary to LHD group, but we presume they had PH because increased pulmonary vascular resistance, since we excluded the possibility of pulmonary hyperflow.

Still considering that the formation of NO can occur because of an inflammatory stimulus, the complete blood counts of dogs with NO measured were analyzed individually at T1 and T2, searching for imbalance in blood cells, especially related to red blood cells and total leukocytes. However, we could not have evaluated if there was direct relationship with the presence of anemia or polycythemia, leukocytosis or leukopenia with the NO values obtained, since few dogs had this kind of variation, but that dogs there was no pattern of increase or decrease in the NO value in the presence of changes in the complete blood count at individually comparison. Nevertheless, we consider that further investigation of the agents involved in more specific inflammatory processes in the body, such as TNF alpha and interleukins (de Laforcade *et al*., 2003; Fahim *et al*., 2004), may be targets of future research involving dogs with PH, since it was not included in the objectives of this study.

To understand the role of nitric oxide in PH in dogs, it is necessary to comprehend the process of establishment and development of PH in this species, which is not yet fully elucidated, although the consensus published by Reinero *et al*. (2020) guide future studies. It is known that physiologically there is a delicate and precise regulation in the balance between vasoconstrictor and vasodilator molecules and that, in PH, this balance is out of adjustment and favors vasoconstriction (Tabima *et al*., 2012). However, after the ACVIM consensus (Keene *et al*., 2019), Reinero *et al*. (2020) published the PH guidelines that defined that the increase in pulmonary arterial pressure (PAP) in dogs may not originally start with the increase in PVR in several situations, such as in increased cardiac output, left-to-right shunts, and increased capillary pressure secondary to LHD. Likewise, the increase in PAP can also occur due to increased PVR or by situations such as pulmonary vascular injury secondary to several causes. Thus, it was recommended by the consensus that the increase in PAP associated with the increase in capillary pressure, determined echocardiographically by the increase in pressure in the left atrium (LA), is called post-capillary PH. The increase in PAP

associated with the increase in PVR without increasing LA pressure, is called pre-capillary PH. And it was also well delineated that an increase in PVR can develop in cases of PH secondary to chronic LHD, being called combined pre and post capillary PH (Reinero *et al*, 2020).

In view of such variety in the definition and classification of PH and considering that the most frequent disease in the clinical routine of the veterinary hospital where this research was developed is MMVD, we chose in this study to simplify the classification of the evaluated dogs. It was based only on the characterization of clinical signs and echocardiographic findings that are known to characterize left cardiac remodeling and possible presence or absence of venous congestion to determine dogs with PH secondary to LHD. Thus, dogs with suspected PH and which did not meet the LHD criterion were classified into dogs with PH not secondary to LHD. In the same way that it was presented in this study, the results showed that the dogs showed differences when comparing echocardiographic parameters of evaluation on the left side. The LA/Ao ratio and the LVIDDN were the most important to characterize the left cardiac remodeling and the E wave and aortic flow velocity to characterize signs of increased preload (Keene *et al*., 2019). This information is relevant to demonstrate that there is a difference in the underlying cause of PH in the dogs evaluated and to justify the possible process of establishing PH in a chronic and slow way in both groups and that in these patients the plasma NO concentration will be able to differentiate dogs with pre-capillary and post-capillary PH.

Analyzing the echocardiographic data and the NO concentration more broadly, it was observed that some echocardiographic variables were more efficient in association with NO in characterizing patients with PH secondary or not to LHD. The variables AT/ET and PA/Ao are indicative of changes in the pulmonary artery, of accelerated pulmonary flow and pulmonary trunk dilation. Both when increased are indicative of suspected PH (Reinero *et al*., 2020) and proved to be important tools for identifying dogs with suspected PH in this study. RV dilation and hypertrophy are also indicative of suspected PH, according to the consensus published by Reinero *et al*. (2020), nevertheless, the consensus did not make it clear which measures can be used for such inference. In the present study, the variables indexed RVILS and RVIDD/LVIDD indicate RV dilation and hypertrophy, respectively, and were important for identifying the probability of PH in the dogs evaluated.

Regarding the echocardiographic criteria recommended by the PH consensus (Reinero *et al*, 2020) to characterize the probability of PH, our study showed that NO was not correlated with these variables that could be indicative of RV remodeling and/or dysfunction and alteration of the pulmonary artery (data not shown). Although the result showed a tendency for the greater availability of NO the higher the probability of PH, the difference was not significant, and we consider the short number of cases evaluated. Despite that, this could probably be due to what has already been discussed about the broad activity of NOS and alternative routes for NO production. In addition, there may also have been an influence of other important mediating factors in the establishment of the pulmonary arterial vasoconstriction process not covered in this research, but also mentioned previously. Among these, endothelin-1 plays a key role in the vasoconstriction process, and it has already been demonstrated by Tessier-Vetzel *et al*. (2006) that, in dogs with spontaneous heart disease and respiratory disease, endothelin-1 is increased in relation to healthy dogs and concentration increases as the heart failure class (International Small Animal Cardiac Health Council classification, 1999) and the severity of the lung disorder worsen.

Moreover, another very important result of Tessier-Vetzel *et al*. (2006) study is that endothelin-1 was positively correlated with echocardiographically estimated systolic pulmonary hypertension in these dogs. Given this, it is possible that NO has a similar behavior in the progression of PH, but it was not possible to evidence it in this research due to limitations such as the number of animals selected or also to the non-dosage of other important mediators that can influence the bioavailability of NO and that could elucidate more details the relationship between it in the severity of PH.

This work has some limitations. One of them is the choice of estimation of NO through the measurement of its substrates in blood, plasma

nitrite and nitrate. Plasma nitrite and nitrate have a longer half-life and may not represent the actual value over time; however, our study showed that there was no significant variation in these substrates in the second dosage after a period. Another possible limiting factor is that this study evaluated the NO concentration in the bloodstream, which may not represent what happens in the pulmonary vascular bed, as it is well known that there is low availability of NO in the lungs at PH. However, a study that was able to assess the pulmonary concentration of NO or NOS in the lungs of spontaneously domiciled dogs affected by PH would be very difficult to be performed due to the low lung biopsy routine, cost to the owner and anesthetic and surgical risks to the patient. Besides that, there is an ethical issue involved in the use of guardian dogs spontaneously affected by PH as an experimental model.

CONCLUSION

In the present study, indirect plasma NO was able to differentiate dogs with PH secondary to LHD from dogs with PH not secondary to LHD and the echocardiographic parameters helped to classify this groups. Nevertheless, NO was not significant to differentiate the low, intermediate, and high probability of PH in dogs, even though, NO demonstrated a tendency to be increased as higher the probability of PH. In these dogs, the NO remained with a similar value between the first and the second collection, showing that there is stability of this substance in the vascular bed.

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