Decrease of nitric oxide and increase in diastolic blood pressure are two events that affect renal function in dogs with pituitary dependent hyperadrenocorticism

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Abstract

Hyperadrenocorticism is a frequent disease in dogs. The excess of circulating cortisol affects different organs and metabolic pathways, producing severe adverse effects that endanger the animal's life. Among these effects, hypertension and renal damage can be mentioned. A group of 20 dogs with pituitary dependent hyperadrenocorticism (PDH) and 12 control dogs were used to study the following parameters: cortisol and nitric oxide (NO *nit/nit*) concentrations, diastolic and systolic blood pressure, renal artery resistance index by Doppler ultrasound, the rate of glomerular filtration by radio-renogram excretion and the presence of proteins in urine. Dogs with PDH showed a significantly lower NO *nit/nit* ($P<0.0001$) than the controls and this correlated with high values of diastolic and systolic pressure ($r = -0.87$; $P<0.0001$ and $r = -0.81$; $P<0.0001$ respectively). Most dogs (80%) are hypertensive mainly due to an increase in diastolic pressure, which correlated positively with the UPC ($r = 0.8$; $P<0.001$) and negatively with the glomerular rate of filtration ($r = -0.58$; $P=0.007$). Systolic pressure only increased in 60% of the cases and did not correlate with the mentioned variables. In PDH the decrease of NO affects blood pressure. The diastolic pressure would seem to have the greatest impact on the kidneys, therefore its evaluation and control are important to avoid and/or control renal damage.

Keywords: Blood pression, Hyperadrenocorticism pituitary dependent, Nitric oxide, Renal function, Scintigraphy.

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Introduction

Hyperadrenocorticism (HAC) or Cushing syndrome (CS) is a frequent disease in dogs characterized by an excess of circulating cortisol. This excess affects various organs and metabolic pathways, producing severe adverse effects that persist even after the disease has been controlled (Haentjens *et al*., 2005; Cabrera Blatter *et al*., 2012).

Among these adverse effects are high blood pressure (HBP) and renal damage (Martiarena *et al*., 2007; Smets *et al*., 2010, 2012b; Shibata and Fujita, 2012) both of which endanger the animal's life. The reasons for HBP due to hyperadrenocorticism are diverse and among them, the following stand out: cortisol activation of the mineralocorticoids' receptors (Ulick *et al*., 1992; Funder and Mihailidou, 2009; Shibata and Fujita, 2012) alteration of microvascular reactivity, endothelial dysfunction, and increase in tone and rigidity of the arterial walls (Haentjens *et al*., 2005; Magiakou *et al*., 2006; Novellas *et al*., 2008; Lien *et al*., 2010; Smets *et al*., 2010). These effects are related to the amount of time with hyperadrenocortisolism before its diagnosis and an adequate treatment is established.

Additionally, this steroid hormone inhibits the nitric oxide endothelial synthase enzyme (eNOS), affecting nitric oxide (NO) concentrations, which are necessary for vasodilatation (Mitchell and Webb, 2002; Yang and Zhang, 2004). Previously, our group proved that in dogs with pituitary dependent HAC (PDH), NO concentrations were decreased and were related to circulatory disturbances of the retina artery and vein, leading to blindness in dogs with PDH (Cabrera Blatter *et al*., 2011). Around 65% of dogs with HAC have HBP, but this percentage can vary according to whether only the systolic pressure (SP) or the diastolic pressure (DP) is considered (Heaney *et al*., 1999). On the other hand, Lien *et al*. (2010) discriminated between those with PDH and those with an adrenal tumor, reporting that in the first, the HBP (evaluating only SP) is present in 20% of the cases and that 52.5% of these dogs already presented microalbuminuria and 32.5% albuminuria, indicative of renal damage. The presence of proteinuria together with HBP has also been reported by other authors in dogs with HAC (Kubota *et al*., 2001; Schellenberg *et al*., 2008; Wehner *et al*., 2008; Smets *et al*., 2012b).

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The kidney is one of the most sensitive organs to an increase in blood pressure (Finco, 2004), and HBP is one of the most important risk factors involved in generating glomerular damage as it leads to alterations of glomerular morphology and function, increasing the intraglomerular capillary pressure (Lees *et al*., 2005; Wehner *et al*., 2008; Cianciolo *et al*., 2016). This favors hyperfiltration by the glomeruli, allowing passage of albumin and, in time, provoking a deterioration of renal function as the filtered protein acts as a nephrotoxic agent (Martiarena *et al*., 2007; Lien *et al*., 2010). Glomeruloesclerosis has been documented in humans and in dogs with Cushing Syndrome (Scholz *et al*., 1957; Oppenheimer and Esterly, 1963; Ortega *et al*., 1996; Waters *et al*., 1997) and is one of the causes of death or of a lower life expectancy.

The objective of this study was to analyze both arterial pressures (diastolic and systolic) and their correlation with NO and cortisol concentrations. Furthermore, evaluate if there is a correlation between these variables and the alteration of the different parameters indicative of renal function or health (proteinuria, glomerular filtration, renal artery resistance index, urine density). Lastly, analyze which of all the above-mentioned variables are clinically useful as indicators of incipient renal damage.

Materials and Methods

Population under study

Population with PDH

Twenty dogs with PDH were studied (11 females and 9 males; of which, 10 were mixed breed, 3 Poodles, 3 Beagles, 2 Golden Retrievers, 1 Daschund and 1 Pit Bull). The age range was between 4 and 15 years old (median: 9.9 years) and their weight between 3.1 and 35 kg (median 11.9 kg). All dogs were diagnosed at the Endocrinology Unit of the Teaching Hospital of the Faculty of Veterinary Sciences of the University of Buenos Aires, where they had been referred by colleagues from private practices.

The dogs showed the characteristic signs of Cushing Syndrome: polydipsia, polyuria, polyphagia, thin inelastic skin, hair loss, large abdomen, weight gain. The diagnosis was confirmed using the cortisol:creatinine relationship in urine before and after oral administration of dexamethasone (Galac *et al*., 1997), ACTH stimulation (basal cortisol measurements and 1 h after intravenous administration of 0.25 mg ACTH), plasma ACTH measurements, abdominal ultrasonography to evaluate the morphology and size of the adrenal glands, and Magnetic Resonance of the sellar region (Gallelli *et al*., 2010; Kooistra and Galac, 2012; Miceli *et al*., 2014).

None of these patients had concurrent diseases (heart disease, clinically evident kidney disease, urinary infections, diabetes mellitus, neoplasia nor systemic infectious diseases) nor were they receiving or had received treatment for PDH prior to this diagnosis. Once diagnosis was carried out and after receiving the owner's written informed consent accepting to allow their pet to participate in the study, the planned studies were carried out.

Control population

A population of 12 healthy dogs (6 neutered females and 6 non-castrated males; median age, 6 years old, range: 3-10 years) from the Faculty of Veterinary Sciences of the Buenos Aires University kennels, on whom the same determinations were carried out as on the PDH population, save for the radio-renogram excretion (by stipulation of the Committee for the Use and Care of Laboratory Animals (CICUAL) of the Faculty of Veterinary Sciences of the University of Buenos Aires), using in this case, standardized values from previous studies as a reference. With the aim of minimizing changes in the values due to stress, all handling for obtaining data was carried out at the dog kennel facilities, where the animals were familiar with their surroundings.

Studies

The procedures described for the PDH population, carried out in a specially conditioned area within the Teaching Hospital, were carried out in the same way for both populations. No sedatives were used, to avoid changes in blood pressure or blood flow in the Doppler study. The owner was present all the time and in contact with their dog.

Blood pressure measurements

At the time of consultation, both systolic and diastolic blood pressure was measured in all dogs using a high definition oscillometric method (VetHDO monitor MD/90, Systeme & Beratung, Germany). The procedure was carried out after 30 minutes of the animal becoming familiar with his surroundings (place, voices, stretcher and personnel) and was relaxed. The dog was positioned in sternal recumbence on the stretcher, a cuff was placed on the left front leg (brachial cephalic artery) and 4 consecutive measurements were taken so the dog could adapt to the pressure of the cuff and the noise of the sphygmomanometer during the first measurements. The last measurement was taken as the reference value. Hypertension was defined as SP > 160 mmHg and/or DP > 90 mmHg (Bodey and Michell, 1996; Brown *et al*., 2007).

Renal Doppler ultrasound study (US-Doppler)

After measuring blood pressure and thirty minutes prior to the study (to recover from the stress of the handling) the area of renal projection was shaved and the animal was positioned on the stretcher. After resting, gel was placed over the area and the study was conducted, always by the same operator. The animals were positioned in lateral recumbence, both left and right, to evaluate the corresponding kidneys using a MINDRAY

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M5VET equipment, with a micro-convex multi-frequency 3-7.5 MHZ transducer. First the morphology of both kidneys was observed, and if alterations such as calcifications, hydronephrosis, cysts, parenchymal changes, atrophy or signs of degeneration were noted, they were excluded from the study. Subsequently, the renal artery resistance index (RI) of each kidney was evaluated using the Doppler and a RI ≤ 0.7 was considered normal (Morrow *et al*., 1996; Novellas *et al*., 2007, 2008).

Radio-renogram excretion in gamma camera scintigraphy

With the aim of evaluating glomerular filtration a dynamic scintigraphic study in gamma camera (radio-renogram excretion) was carried out in a planar gamma camera. The radioisotope used was ^{99m}Tc (technetium 99 metastable sodium pertectneciate, DTPA CaNa$_3$-Sn RADIOFARMA® TC, 3-4mCi/111-148 Mbq) at a fixed distance of 26 cm over the center of the gamma camera. Firstly, static images were taken of the syringes with the radiation tracer (before and after intravenous bolus injection) and of the patient in lateral recumbence, to measure renal depth and subsequently calculate glomerular filtration. Afterwards, the animal was placed in ventral recumbence and with the gamma camera centered dorsal to the kidneys. Dynamic images were collected every 15 seconds in a digital 64 x 64 x 16 mold, for 15 minutes. Sedation of the dogs was not necessary to carry out the study.

Two parameters were determined for this study: 1) the rate of glomerular filtration (GFR); 2) the initial reactivity time (RT) for each kidney (point at which the curve reached the highest activity after implementing the radiation tracer, indicating the maximum time for glomerular filtration).

The phases of perfusion, glomerular filtration and elimination of the radiation tracer were assessed by processing the images and obtaining the glomerular function curve. It is considered that for a healthy kidney, the GFR should be >3 ml/kg/min and that values between 1.2 and 2.7 ml/kg/min and azotemia values of <1.2 ml/kg/min are indicative of subclinical renal insufficiency. A normal RT is considered to be lower than 3.5 minutes, with values higher than 5 minutes corresponding to kidneys with altered function, and intermediate values between 3.6 and 4.9 minutes, considered suspect (Bartges and Polzin, 2013).

Biochemical studies

Blood and urine samples for all the biochemical studies were obtained the day after the aforementioned studies. After the serum was obtained from the blood samples by centrifugation and stored at -80 ℃ until its processing, urea, creatinine, total proteins, albumin, nitric oxide (NO) and cortisol were evaluated. Blood samples were taken after a 12 h fast and collected in plastic test tubes without anticoagulant. Urea, creatinine, total proteins and albumin were all measured in an automated equipment (Metrolab 2100), using a standardized enzymatic-colorimetric method with specific reagents and according to the manufacturer's indications.

Urine samples were obtained immediately after blood sampling, using a catheter and a sterile jar. A complete physical and chemical analysis was carried out, considering densities >1025 to be normal for dogs with a standard liquid intake and normal urination according to their weight, and a quantitative and qualitative evaluation of the proteins present was also carried out. Protein quantification was accomplished using the Protein/Creatinine ratio in urine (UPC), considering values lower or equal to 0.3 to be normal, between 0.31 and 0.49 are suspect and values greater than 0.5 clearly pathological (Martiarena *et al*., 2007). For the qualitative determination of proteins, each fraction was separated by molecular weight (MW) using a polyacrylamide gel in the presence of dodecyl sodium sulphate (SDS-PAGE method) with rapid argentum coloring. A light band of proteins of 69 kd MW (20 mg/L) was considered physiological. Bands ≥ 69 kd MW were considered indicative of proteins of glomerular origin and bands <69 Kd MW were considered proteins of tubular origin (Marshall and Williams, 1998; Zini *et al*., 2004; Martiarena *et al*., 2007).

Cortisol and nitric oxide determinations

Serum cortisol was evaluated using ELISA ("Cortisol ELISA, ABCAM"), with an intra and inter assay coefficient of variation of 4.8 and 7.8 respectively and a sensitivity of 0.4 µg/dL. The reference range obtained by our laboratory from a control population was of 0.8-4.5 µg/dL (median: 2.1 µg/dL).

NO was measured as previously described by Cabrera Blatter *et al.* (2012), using ELISA (BioVision, EE.UU) and calculating the NO production by the nitrites and nitrates produced (NO *nit/nit*). The intra and inter assay coefficients of variation were 3.1% and 5.8% respectively with a sensitivity of 0.1 nmol nitrites/well. The reference range for our laboratory is 3.2 – 7.2 nmol/mL (median: 5 nmol /mL).

Statistical analysis

After carrying out the Pearson Omnibus Normality test, and given that most of the variables under study showed a non-parametric distribution, the results are expressed as median (Mn) and ranges (minimum and maximum values) and the medians between the PDH and the control groups were compared using the Mann-Whitney test. Spearman's test was used to evaluate correlation between the different variables and Fisher's Exact test was used to evaluate the existence of an association between blood pressure, UPC and the resistance of the renal artery, calculating the Odds Ratio

(OR) (software GraphPad 6, USA). Significance was set at P<0.05.

Animal welfare

The Committee for the Use and Care of Laboratory Animals (CICUAL) of the Faculty of Veterinary Sciences of the University of Buenos Aires approved all procedures (Protocol 20720130100004BA) and a written consent was obtained from all proprietors for carrying out all evaluations and their subsequent publication.

Results

Blood pressure and renal artery resistance index (RI)

Eighty percent (16/20) of the dogs studied presented diastolic hypertension, with 20% (4/20) showing <90 mmHg. SP was high (systolic hypertension) in 60% (12/20) of the animals and was <160 mmHg in the remaining 40% (8/20). All dogs with high SP also had a high DP, while of the 8 cases with a normal SP, 5 had a high DP. Values of both pressures were significantly higher in dogs with PDH as compared to the control group (P=0.004 and P=0.002, for SP and DP respectively) (Fig.1).

The RI of the left renal artery was high (Me = 0.68; range 0.5-.091) in 40% (8/20) of the cases and the RI of the right renal artery was high (Me = 0.71, range 0.57-0.93) in 50% (10/20) of the cases, without showing significant differences between them. In the control group, RI was <0.70 in both renal arteries (Me = 0.5, range 0.35-0.6), showing significant differences with the HAC-PD group (P<0.001).

Radio-renogram excretion

No dog presented severe deterioration of renal function, although a decrease in kidney mass was observed in 25% (4/20; 3 of the left kidney and 1 of the right), coinciding with affected values of GFR and RT (Fig. 2). GFR values (Fig. 3) were between 1.2 and 2.7 ml/kg/min in 35% (7/20) of the dogs and was normal (> 3 ml/kg/min) in 65% (13/20) of the dogs. RT was altered (>3.5 and <5 minutes) in 35% (7/20) of the dogs, coinciding with those in which the GFR was also altered (Fig. 3).

No differences in RT were observed between the left and right kidneys. According to the analysis of the radio-renogram curves, the phases of glomerular filtration and renal perfusion were affected but the excretion phase was normal.

Serum biochemical studies

Urea, creatinine, total proteins and albumin were within the normal reference values in serum for our laboratory in all cases (Table 1) and no significant differences were found with the control group. Plasma concentrations of cortisol were significantly higher (P<0.0001) in dogs with PDH (Table 1 and Fig. 4). On the other hand, NO *nit/nit* concentrations were significantly lower (P<0.0001) in animals with PDH (Table 1 and Fig. 4).
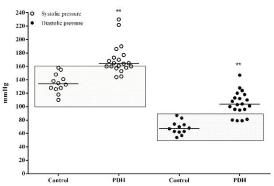

Fig. 1. Systolic (SP) and diastolic (DP) arterial pressure in control and PDH dogs. The shaded area indicates the area of normal SP and DP arterial pressure. The full line indicates the median and each point represents an evaluated dog. (SP: **P=0.004 vs. control) (DP: **P=0.002 vs. control).
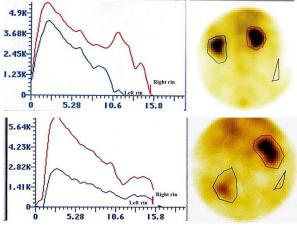

Fig. 2. Data of the gamma camera scintigraphy and radio-renogram excretion in two dogs with PDH. Upper panel: Study within normal parameters. In the figure on the left, the glomerular filtration curves (GRF) can be observed; passage and excretion, being similar for the evaluation times and the RT is also similar (peak of the curve). In the figure on the right, both kidneys with equal functional parenchyma uptake of the radioisotope can be observed. Bottom panel: Study of a dog with affected kidney function. Note that the filtration curve and RT are lower than those of the right kidney (see explanation in the text) and left kidney (right side) has less functional parenchyma (less uptake of the radioisotope).

Urine

Urine density was significantly lower (P<0.05) in the PDH group as compared to the control, with values <1025 in 60% of the dogs (12/20) and higher values in the remaining 40% of the dogs. All 20 dogs presented with polyuria and polydipsia at the time of the study. In the control group, urine density was >1025 in the 12 dogs included in the study. The rest of the physical and chemical studies showed no alterations in either group. UPC was significantly higher in the PDH group as compared to the control, with high values (>0.5) in 55% (11/20) of the dogs, unspecific values in 10% (2/20) dogs and less than 0.3 in 35% (7/20) (Table 1).
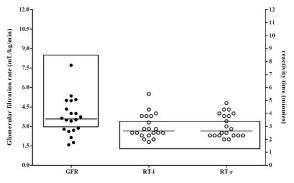
Fig. 3. Data of the glomerular filtration rate (GFR) and reactivity time in dogs with PDH. The box indicates the normal area for GFR and RT. The whole line represents the median and each circle is an individual patient that was evaluated.

Table 1. Urine density, urea, creatinine and UPC, cortisol and NO *nit/nit* values in control and HAC-PD dogs.

Parameter	HAC-PD	Control	P
Urea (mg/dL)	37 (20-60)	42 (30-57)	NS
Creatinine (mg/dL)	0.77 (0.5-1.2)	0.69 (0.4-0.9)	NS
Total proteins (g/L)	6.8 (5.7-7.3)	7.1 (5.6-7.2)	NS
Albumin (g/L)	3.2 (2.8-3.3)	3.1 (2.9-3.3)	NS
Urine density	1,014 (1,005-1,038)*	1,027 (1,022-1,035)	<0.05
UPC	0.95 (0.11-5.8)***	0.19 (0.1-0.28)	<0.0001
Cortisol (µg/dL)	5.6 (2.8-10.6)***	2.1 (0.8-4.5)	<0.0001
NO *nit/nit* (nmol/mL)	1.54 (0.04-3.7)***	5 (3.2-7.2)	<0.0001

Values are expressed as median and ranges (minimum and maximum).

A 65% (13/20) of the dogs presented glomerular proteinuria, coinciding with those presenting high or unspecific UPC values. No animal showed proteinuria of tubular origin. In the control group, UPC was <0.3 with no evidence of proteinuria in any of the 12 cases.

Correlation and association analysis

Plasma cortisol and NO *nit/nit* showed a significant inverse correlation (r = -0.67; P<0.0001) (Fig. 5).

SP correlated positively with cortisol (r = 0.46; P=0.008) and negatively with NO (r = -0.87; P <0.0001) (Fig. 6). No correlation (r = 0.3; P=0.2) nor association (P=1.00) was observed between SP and UPC. Nor was a correlation observed between SP, the renal artery resistance (r = -0.05; P=0.6) and the GFR (r = -0.32; P=0.16).

DP correlated positively with plasma cortisol (r = 0.63; P<0.0001) and inversely with NO *nit/nit* (r = -0.81; P<0.0001) (Fig. 6).
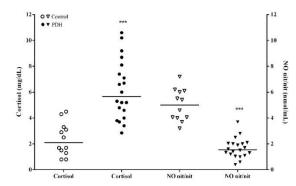

Fig. 4. Plasma cortisol and NO *nit/nit* concentrations in control dogs and in dogs with PDH. Each circle represents a dog, open circles and triangles: controls and filled-in circles and triangles: dogs with PDH; the full line represents the median. *** P<0.0001, PDH vs. controls.
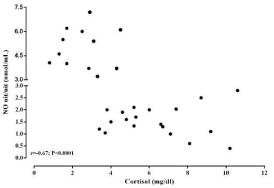

Fig. 5. Correlation between NO *nit/nit* and plasma cortisol. Note that the 12 control dogs have the highest NO *nit/nit* values (top left quadrant) to then decrease as the cortisol concentrations increase.

Correlation was also observed with UPC (r = 0.8; P <0.001) (Fig. 7), and a significant association (P <0.05) was found between the presence of proteinuria and a high DP, with an OR of 0.08 (95%CI: 0.01-0.63). No significant correlation was seen between DP and the renal artery resistance index (r = 0.35; P=0.16), and DP showed a negative correlation with GFR (r = -0.58; P=0.007) (Fig. 6). The RI correlated positively with plasma cortisol (r = 0.69; P=0.0007) and negatively with NO *nit/nit* (r = -0.53, P=0.014) (Fig. 8). The increase in RI coincided with an increase in DP in 10/20 dogs, however no statistically significant association was observed between these parameters (P=0.36). Regarding SP, 40% (8/20) of the dogs coincided in having both parameters altered, though this association was not significant either (P=0.36). No correlation was observed between RI and UPC (r = 0.2; P=0.8), although these parameters did show a statistical association (P <0.01), where all 11 dogs with UPC >0.5 and the two dogs with unspecific values had a RI >0.7, with an OR of 0.017 (95% CI: 0.001-0.41). Correlation was found between NO *nit/nit* and GFR (r = -0.34; P=0.14).
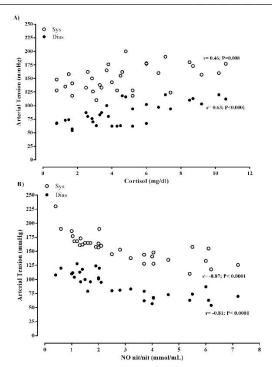
Fig. 6. Correlation between the systolic and diastolic pressures and plasma cortisol (A) and with NO *nit/nit* (B). It can be seen how the diastolic pressure is the most affected by the cortisol concentrations (A), contrary to what happens with the NO *nit/nit* (B), where both arterial pressures are similarly affected.
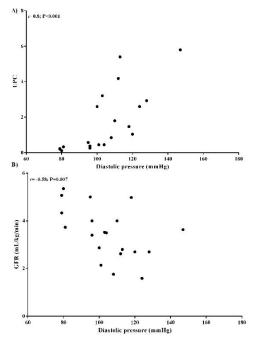

Fig. 7. Correlation between UPC (A) and GFR (B) with diastolic pressure. The elevated presence of proteins in urine in the PDH clearly coincides with high diastolic values (A). The glomerular filtration rate is also affected by the diastolic pressure (B), although to a lesser degree given the correlation values.
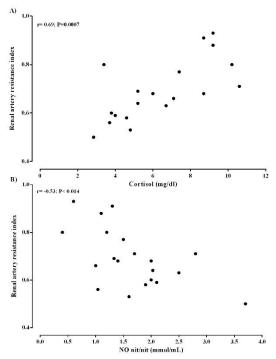

Fig. 8. Correlation between the RI vs. plasma cortisol (A) and NO *nit/nit* (B). Evidently the alteration of this index in the PDH is influenced mainly by the cortisol concentrations (A) and to a lesser degree by the NO (B) as shown by the correlation values.

Finally, GFR did not show correlation with cortisol (r = -0.16; P=0.5), although the aforementioned hormone correlated with NO *nit/nit* (r = 0.54; P=0.004).

Discussion

Among the adverse effects of the chronic hypercortisolism of PDH is HBP and, as a consequence, renal glomerular dysfunction. Studies in human medicine have shown that these adverse effects persist even after reaching normal cortisol concentrations (Haentjens *et al*., 2005), coinciding with studies in dogs (Martiarena *et al*., 2007; Smets *et al*., 2012a), thus early identification is important to implement adequate preventive or therapeutic measures.

In the first place, it is necessary to analyze the behavior of NO *nit/nit* in PDH as it is one of the triggering factors for HBP and glomerular damage, together with and because of the excess cortisol. As plasma cortisol concentrations increase, NO *nit/nit* descends, a fact which has already been reported both in dogs and in humans with Cushing syndrome (Wen *et al*., 2000; Cabrera Blatter *et al*., 2012) and is because of the inhibition of eNOS (Magiakou *et al*., 2006). Therefore, the descent of NO in PDH, together with other proposed mechanisms (Haentjens *et al*., 2005; Magiakou *et al*., 2006; Novellas *et al*., 2008; Lien *et al*., 2010; Smets *et al*., 2010), would be one of the causes

of HBP, as evidenced by the strong correlation observed between NO *nit/nit* and both blood pressures. Various authors refer that between 20 and 86% of dogs with PDH have high blood pressure (Ortega *et al*., 1996; Hurley and Vaden, 1998; Novellas *et al*., 2008; Lien *et al*., 2010), considering one or both types of blood pressure and the method of measuring them.

In this study, where both arterial pressures were evaluated, 80% of the dogs had hypertension and the DP was a determining factor in this. It is noteworthy that this pressure was the only one that was elevated in 20% of the animals studied, with a normal SP. We did not observe the inverse situation: high SP with normal DP. If only SP had been taken into consideration, only 60% of the dogs would have been hypertensive, hence it is very probable that its increase is later on, as the disease progresses and more vasodilator mechanisms are affected (Novellas *et al*., 2008; Wehner *et al*., 2008) and for this reason, in addition to the evaluation method used and that SP was the only pressure measured, Lien *et al*. (2010) reported only 20% as being hypertensive. Although both blood pressures correlated with NO *nit/nit,* when analyzing their correlation with cortisol, we see that the impact of the hormone is greater on the DP than on SP. Therefore, one could assume that DP would be affected earlier and to a greater extent than the systolic, by the combined action of the excess of cortisol and the descent of NO, thus one could consider that dogs with PDH are eminently diastolic hypertensive.

Diastolic hypertension was associated with the presence of proteinuria, evaluated using UPC (an indicator of glomerular damage), showing correlation between these parameters and becoming a risk factor. It also showed an association and correlated with the decrease in GFR. We have not found any other similar reports in dogs. Wehner *et al*. (2008) found a correlation between UPC and SP in dogs affected by several diseases that produce hypertension without differentiating diseases (mentioning only two cases with endocrine pathology), and without evaluating DP. In this study, we only analyzed dogs with PDH, hence our findings cannot be made extensive to cases of adrenal tumors or other endocrine pathologies. Due to all the above, it is important to take into consideration the DP, as if it is not rapidly controlled, its increase will start to damage the glomeruli, allowing filtration of high molecular weight proteins and affecting the rate of glomerular filtration (Lees *et al*., 2005; Wehner *et al*., 2008; Cianciolo *et al*., 2016). Given the correlation between both blood pressures with cortisol and NO *nit/nit*, and of DP with UPC and GFR, it is to be expected that dogs recently diagnosed with PDH are already hypertensive and have some degree of glomerular damage. Suspicion of renal glomerular damage is reinforced by the findings in the RI of the renal artery. This index was found to be altered in 40-50% of the cases (according to the kidney evaluated) and correlated with cortisol and NO *nit/nit*, but not with the blood pressures. Excess cortisol increases the renal vascular resistance in both in humans and dogs with HAC (Magiakou *et al*., 2006; Smets *et al*., 2010). In addition, both cortisol and NO will affect the renal artery by the previously discussed mechanisms. In human medicine, Ingaramo (2003) report a correlation between RI and systolic but not diastolic pressure, however our results only coincide regarding the lack of correlation with DP and differ in that in dogs SP did not correlate either, hence this needs further study. Fifty percent of the dogs with diastolic and systolic hypertension had an elevated RI, so presumably in these cases there already is an important amount of damage to the renal parenchyma and the internal vasculature (Morrow *et al*., 1996; Petersen *et al*., 1997; Novellas *et al*., 2008). It is probable that renal parenchyma is progressively affected according to the amount of time of evolution of PDH and the untreated hypertension; hence, with an RI <0.7, renal damage should not be discarded and is indicative that the kidney can still recuperate. On the contrary, RI >0.7 values associated with HBP and UPC>0.5, have a reserved prognosis (Wehner *et al*., 2008).

This slow but progressive evolution toward renal damage would explain the association between RI and UPC but that they are uncorrelated. Prior to detection of high molecular weight proteins (analyzed by UPC) microalbuminuria can already be detected due to the incipient glomerular damage (De Jong and Gansevoort, 2009; Lien *et al*., 2010). This microfiltration of proteins slowly affects renal parenchyma (Martiarena *et al*., 2007; Shibata and Fujita, 2012), however, it is still not enough to be reflected in the US-doppler studies (as the RI<0.7) nor by the radio-renogram excretion (as GFR >3ml/kg/min). Due to methodological reasons, we did not study the microalbuminuria in this study, so there are probably a larger number of affected individuals. According to the study by Lien *et al.* (2010), microalbuminuria is present in 52.5% of dogs with PDH, hence one should consider that at the moment of evaluating RI and UPC there already is glomerular and renal parenchyma damage in dogs with PDH. In the present study, 65% of the cases had a UPC value >0.3, jointly considering those with unspecific UPC (10%) and those with values >0.5 (55%), with albumin of glomerular origin being the predominant protein present according to the electrophoretic urine proteinogram (Martiarena *et al*., 2007) and in addition the dogs presenting hypertension. These data contrast with those of Lien *et al*. (2010) for whom only 32.5% of the dogs show albuminuria, indicating a more advanced stage of glomerular damage. This discrepancy could be, on the one hand, because the

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animals in both studies were at different stages of evolution of PDH (longer in our case) and, on the other hand, if we consider the microalbuminuria, the data are similar, hence UPC and the electrophoretic proteinogram with SDS-PAGE would be including the microalbuminuria in the case of the dog. To summarize, in the case of diastolic hypertension and UPC with values already higher than 0.3, it is highly probable that the glomerulus is compromised and it is advancing toward parenchymal damage. In these cases, the RI value will be of use as an indicator of the state of the kidney parenchyma.

Regarding the radio-renogram excretion, no dog presented severe functional deterioration, although in 25% one kidney was more affected than the other. As mentioned before, GFR was inversely affected by the NO *nit/nit* concentrations. Possibly as a reflection of the lower vasodilation of the afferent and efferent small arteries, provoking a decrease in renal plasmatic flow (Denton *et al*., 2001). Although administration of glucocorticosteriods increases GFR in the short term (Wen *et al*., 2000), chronic exposition, as is the case of HAC, provokes the opposite effect (Haentjens *et al*., 2005; Smets *et al*., 2010) as there is a long-term counter-productive effect of cortisol on GFR that persists even in HAC individuals in remission. Considering that most (65%) of the dogs studied presented a normal GFR, despite already showing proteinuria and an affected RI, this would indicate that filtration is not affected early on and that the polyuria typical of PDH would not be due to a greater filtration, but mainly because the vasopressin hormone is affected (Baas *et al*., 1984; Knoepfelmacher *et al*., 1997). Both GFR and RT did not correlate with cortisol (at least directly) and this could be explained by the hypothesis postulated by Haentjens *et al*. (2005). Our study evaluated cortisol, GFR and RT at a precise moment in time and not prospectively, a fact which could explain the lack of correlation. Regarding the other indicators of renal function that were analyzed (urine density, urea and creatinine) Feldman and Nelson (2004) describe in dogs with HAC that 85% present urine densities <1020. Our data are close to this value (60% of the cases). The fact that urine density is not noticeably low (<1010), despite the polyuria-polydipsia, should be attributed to the important proteinuria observed. It is necessary to highlight that the concentrations of urea and creatinine were within the reference range for our laboratory, coinciding with data published by Feldman and Nelson (2004) in dogs with PDH. These authors postulate that it could be due to subclinical states of renal disease, as urea and creatinine concentrations increase when the disease is more advanced. Our observations in the radio-renogram excretion study would explain the normal urea and creatinine concentrations and support Feldman and Nelson's hypothesis.

Conclusions

It is clear that PDH hypercortisolism leads, firstly, to the decrease of NO, which affects the diastolic pressure before the systolic, being eminently a diastolic hypertension. The hypertension together with the excess of cortisol progressively damage the glomeruli and the renal parenchyma, resulting in the presence of proteins in urine, alteration of RI and finally the decrease of GFR and increase of RT, affecting, in some cases, the functional mass of one or both kidneys.

Thus, measuring diastolic arterial pressure together with RI and UPC are the earliest indicators of glomerular damage which, if not treated, end in renal function being greatly affected.

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References

Baas, J.J., Schaeffer, F. and Joles, J.A. 1984. The influence of cortisol excess on kidney function in the dog. Vet. Q. 6, 17-21.

Bartges, J. and Polzin, D.J. 2013. Centellografia Renal, in: Nefrología Y Urología de Pequeños Animales. Tomo 1, pp: 164-175.

Bodey, A.R. and Michell, A.R. 1996. Epidemiological study of blood pressure in domestic dogs. J. Small Anim. Pract. 37, 116-125.

Brown, S., Atkins, C., Bagley, R., Carr, A., Cowgill, L., Davidson, M., Egner, B., Elliott, J., Henik, R., Labato, M., Littman, M., Polzin, D., Ross, L., Snyder, P. and Stepien, R. 2007. Guidelines for the Identification , Evaluation , and Management of Systemic Hypertension in Dogs and Cats. J. Vet. Intern. Med. 21, 542-558.

Cabrera Blatter, M., del Prado, A., Galleli, M., D'Anna, E., Ivanic, J., Esarte, M., Miceli, D., Gómez, N. and Castillo, V. 2011. Blindness in dogs with pituitary dependent hyperadrenocorticism: Relationship with glucose, cortisol and triglyceride concentration and with ophthalmic blood flow. Res. Vet. Sci. 92, 387-392.

Cabrera Blatter, M., del Prado, B., Miceli, D., Gomez, N., Ivanic, J., Di Tollo, B., Galleli, M. and Castillo, V. 2012. Interleukin-6 and insulin incrase and nitric oxide and adiponectin decrease in blind dogs with pituitary-dependent hyperadrenocorticism. Res. Vet. Sci. 93, 1195-1202.

Cianciolo, R., Hokamp, J. and Nabity, M. 2016. Advances in the evaluation of canine renal disease. Vet. J. 215, 21-29.

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De Jong, P. and Gansevoort, R. 2009. Focus on microalbuminuria to improve cardiac and renal protection. Nephron. Clin. Pr. 111, 204-211.

Denton, K., Li, M., Anderson, W. and Whitworth, J. 2001. Glomerular hypertension and hyperfiltration in adrenocorticotrophin-induced hypertension in rats: the role of nitric oxide. J. Hypertens. 19, 327-334.

Feldman, E. and Nelson, R. 2004. Canine hyperadrenocorticism (Cushing's syndrome), Canine and Feline Endocrinology and Reproduction. St. Louis.

Finco, D. 2004. Association of Systemic Hypertension with Renal Injury in Dogs with Induced Renal Failure. J. Vet. Intern. Med. 18, 289-294.

Funder, J. and Mihailidou, A. 2009. Aldosterone and mineralocorticoid receptors: Clinical studies and basic biology. Mol. Cell. Endocrinol. 301, 2-6.

Galac, S., Kooistra, H., Teske, E. and Rijnberk, A. 1997. Urinary corticoid/creatinine ratios in the differentiation between pituitary-dependent hyperadrenocorticism and hyperadrenocorticism due to adrenocortical tumour in the dog. Vet. Quart. 19, 17-20.

Gallelli, M., Cabrera Blatter, M. and Castillo, V. 2010. A comparative study by age and gender of the pituitary adenoma and ACTH and alpha-MSH secretion in dogs with pituitary-dependent hyperadrenocorticism. Res. Vet. Sci. 88, 33-40.

Haentjens, P., De Meirleir, L., Abs, R., Verhelst, J., Poppe, K. and Velkeniers, B. 2005. Glomerular filtration rate in patients with Cushing's disease: a matched case-control study. Eur. J. Endocrinol. 153, 819-829.

Heaney, A., Hunter, S., Sheridan, B. and Atkinson, A. 1999. Increased pressor response to noradrenaline in pituitary dependent Cushing's syndrome. Clin. Endocrinol. (Oxf). 51, 293-299.

Hurley, K.J. and Vaden, S.L. 1998. Evaluation of urine protein content in dogs with pituitary-dependent hyperadrenocorticism. J. Am. Vet. Med. Assoc. 212, 369-373.

Ingaramo, R. 2003. El eco-Doppler renal y su utilidad en la hipertensión arterial. Rev. Fed. Arg. Cardiol. 32, 487-490.

Knoepfelmacher, M., Pradal, M., Di Dio, R., Salgado, L., Semer, M., Wajchenberg, B. and Liberman, B. 1997. Resistance to vasopressin action on the kidney in patients with Cushing's disease. Eur. J. Endocrinol. 137, 162-166.

Kooistra, H. and Galac, S. 2012. Recent Advances in the Diagnosis of Cushing's Syndrome in Dogs. Top. Companion Anim. Med. 27, 21-24.

Kubota, E., Hayashi, K., Matsuda, H., Honda, M., Tokuyama, H., Okubo, K., Naitoh, M., Arakawa, K. and Saruta, T. 2001. Role of intrarenal angiotensin II in glucocorticoid-induced renal vasodilation. Clin. Exp. Nephrol. 5, 186-192.

Lees, G., Brown, S., Elliott, J., Grauer, G. and Vaden, S. 2005. Assessment and Management of proteinuria in dogs and cats. J. Vet. Intern. Med. 19, 377-385.

Lien, Y., Hsiang, T. and Huang, H. 2010. Associations among systemic blood pressure, microalbuminuria and albuminuria in dogs affected with pituitary- and adrenal-dependent hyperadrenocorticism. Acta. Vet. Scand. 52, 1-6.

Magiakou, M., Smyrnaki, P. and Chrousos, G. 2006. Hypertension in Cushing's syndrome. Best Pract. Res. Clin. Endocrinol. Metab. 20, 467-482.

Marshall, T. and Williams, K. 1998. Clinical analysis of human urinary proteins using high resolution electrophoretic methods. Electrophoresis 19, 1752-1770.

Martiarena, B., Madalena, L., Mira, G., Fidanza, M., Lalía, J., Ortemberg, L., Loiza, M. and Castillo, V. 2007. Evaluación de la proteinuria en perros con Enfermedad de Cushing, previo y posterior al tratamiento con Ácido Retinoico y Ketoconazol. AN. VET. 23, 45-54.

Miceli, D., Cabrera Blatter, M., Gallelli, M., Pignataro, O. and Castillo, V. 2014. Involvement of glucagon-like peptide 1 in the glucose homeostasis regulation in obese and pituitary-dependent hyperadrenocorticism affected dogs. Res. Vet. Sci. 97, 211-217.

Mitchell, B. and Webb, R. 2002. Impaired Vasodilation and Nitric Oxide Synthase Activity in Glucocorticoid-Induced Hypertension. Biol. Res. Nurs. 4, 16-21.

Morrow, L., Salman, D., Lappin, R. and Wrigley, R. 1996. Comparison of the resistive index to clinical parameters in dogs with renal disease. Vet. Radiol. Ultrasound 37, 193-199.

Novellas, R., Espada, Y. and De Gopegui, R. 2007. Doppler Ultrasonographic Estimation of Renal and Ocular Resistive and Pulsatility Indices in Normal Dogs and Cats. Vet. Radiol. Ultrasound 48, 69-73.

Novellas, R., Ruiz de Gopegui, R. and Espada, Y. 2008. Determination of renal vascular resistance in dogs with diabetes mellitus and hyperadrenocorticism. Vet. Rec. 163, 592-596.

Oppenheimer, E. and Esterly, J. 1963. Glomerular lesions in nephrotic syndrome and their relation to cortisone therapy. Bull. Johns Hopkins Hosp. 113, 158-172.

Ortega, T., Feldman, E., Nelson, R., Willits, N. and Cowgill, L. 1996. Systemic arterial blood pressure and urine protein/creatinine ratio in dogs with hyperadrenocorticism. J. Am. Vet. Med. Assoc. 209, 1724-1729.

Petersen, L., Petersen, J., Talleruphuus, U., Ladefoged,

S., Mehlsen, J. and Jensen, H.A. 1997. The pulsatility index and the resistive index in renal arteries. Associations with long-term progression in chronic renal failure. Nephrol. Dial. Transplant. 12, 1376-1380.

Schellenberg, S., Mettler, M., Gentilini, F., Portmann, R., Glaus, T. and Reusch, C. 2008. The effects of hydrocortisone on systemic arterial blood pressure and urinary protein excretion in dogs. J. Vet. Intern. Med. 22, 273-281.

Scholz, A., Sprague, G. and Kernohan, W. 1957. Cardiovascular and Renal complications of cushing's syndrome. N. Engl. J. Med. 256(18), 833-837.

Shibata, S. and Fujita, T. 2012. Mineralocorticoid receptors in the pathophysiology of chronic kidney diseases and the metabolic syndrome. Mol. Cell Endocrinol. 350, 273-280.

Smets, P., Lefebvre, H., Kooistra, H., Meyer, E., Croubels, S., Maddens, B., Vandenabeele, S., Saunders, J. and Daminet, S. 2012a. Hypercortisolism affects glomerular and tubular function in dogs. Vet. J. 192, 532-534.

Smets, P., Lefebvre, H., Meij, B., Croubels, S., Meyer, E., Van de Maele, I. and Daminet, S. 2012b. Long-Term Follow-Up of Renal Function in Dogs after Treatment for ACTH-Dependent Hyperadrenocorticism. J. Vet. Intern. Med. 26, 565-573.

Smets, P., Meyer, E., Maddens, B. and Daminet, S.

2010. Cushing's syndrome, glucocorticoids and the kidney. Gen. Comp. Endocrinol. 169, 1-10.

Ulick, S., Wang, Z., Blumenfeld, D. and Pickering, G. 1992. Cortisol inactivation overload: A mechanism of mineralocorticoid hypertension in the Ectopic Adrenocorticotropin Syndrome. J. Clin. Endocrinol. Metab. 74, 963-967.

Waters, C., Adams, L., Scott-Moncrieff, J., DeNicola, D., Snyder, P., White, M. and Gasparini, M. 1997. Effects of glucocorticoid therapy on urine protein-to-creatinine ratios and renal morphology in dogs. J. Vet. Intern. Med. 11, 172-177.

Wehner, A., Hartmann, K. and Hirschberger, J. 2008. Associations between proteinuria, systemic hypertension and glomerular filtration rate in dogs with renal and non-renal diseases. Vet. Rec. 162, 141-147.

Wen, C., Li, M. and Whitworth, J. 2000. Role of Nitric Oxide in Adrenocorticotrophin-Induced Hypertension: L-Arginine Effects Reversed By N-Nitro-L-Arginine. Clin. Exp. Pharmacol. Phisiol. 27, 887-890.

Yang, S. and Zhang, L. 2004. Glucocorticoids and Vascular Reactivity. Curr. Vasc. Pharmacol. 2(1), 1-12.

Zini, E., Bonfanti, U. and Zatelli, A. 2004. Diagnostic relevance of qualitative proteinuria evaluated by use of sodium dodecyl sulfate-agarose gel electrophosis and comparison with renal histologic findings in dogs. Am. J. Vet. Res. 65, 964-971.