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Original Articles

Calcium, phosphorus, urinary fractional excretion of phosphorus and parathormone in dogs with visceral leishmaniasis

Cálcio, fósforo, fração de excreção urinária de fósforo e paratormônio em cães com leishmaniose visceral

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A R T I C L E I N F O

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A B S T R A C T

The kidneys perform an essential role in the regulation of serum phosphorus concentration. In visceral leishmaniasis (VL), the decline of the glomerular filtration rate leads to hyperphosphatemia, whose primary consequence is the development of secondary renal hyperparathyroidism. This study aimed to determine the concentration of serum phosphorus, ionized calcium (Ca_i), urinary fractional excretion of phosphorus (uFEP), and parathormone (PTH) in azotemic and non-azotemic dogs with VL and evaluate the uFEP as a marker of secondary renal hyperparathyroidism. The study comprised 31 dogs, divided into 24 sick animals and 07 healthy animals used as a control group (GC). The sick animals were classified into groups as azotemic (GA) and non-azotemic (GNA). The serum levels of phosphorus, creatinine, Ca_i, and the plasma levels of PTH were measured and compared between groups. The uFEP was calculated using values of serum and urinary levels of phosphorus and creatinine. The Kruskal-Wallis test with a 0.05 significance level was used, followed by the Mann-Whitney test as a *post hoc* test, with a 0.016 significance level. The levels of serum phosphorus were higher in the GA group, whereas for the PTH, the GNA and GA groups demonstrated high values compared to the GC. The uFEP was significantly higher in the GA when compared to the GNA, although there was no statistical difference between the GC and the GNA. The uFEP showed to be a late marker of chronic kidney disease and secondary hyperparathyroidism in dogs with visceral leishmaniasis.

Palavras-chave:
Excreção de fósforo
Hiperparatireoidismo
Creatinina

R E S U M O

Os rins desempenham um papel essencial na regulação da concentração sérica de fósforo. Na leishmaniose visceral (LV), o declínio da taxa de filtração glomerular leva à hiperfosfatemia, cuja consequência primária é o desenvolvimento de hiperparatireoidismo renal secundário. Este estudo teve como objetivo determinar a concentração de fósforo sérico, cálcio ionizado (Ca_i), excreção urinária fracionada de fósforo (uFEP) e paratormônio (PTH) em cães azotêmicos e não azotêmicos com LV e avaliar o uFEP como marcador do hiperparatireoidismo secundária renal. O estudo envolveu 31 cães, classificados em 24 animais doentes e 07 animais saudáveis utilizados como grupo controle (GC). Os animais doentes foram classificados em azotêmicos (GA) e não azotêmicos (GNA). Os níveis séricos de fósforo, creatinina, Ca_i e os níveis plasmáticos de PTH foram medidos e comparados entre os grupos. A uFEP foi calculada usando-se os valores dos níveis sérico e urinário de fósforo e creatinina. Foi utilizado o teste de Kruskal-Wallis com nível de significância de 0,05, seguido do teste de Mann-Whitney como *post hoc* teste, com nível de significância de 0,016. Os níveis de fósforo sérico foram maiores no grupo GA, enquanto para o PTH os grupos GNA e GA demonstraram valores elevados em relação ao GC. O uFEP foi significativamente maior no GA quando comparado ao GNA, embora não ocorra diferença estatística entre o GC e o GNA. O uFEP mostrou ser um marcador tardio de doença renal crônica e hiperparatireoidismo secundário em cães com leishmaniose visceral.

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INTRODUCTION

Canine visceral leishmaniasis is a complex disease of high prevalence, which may reach up to 63% of the canine population in endemic areas. This infirmity causes a chronic inflammatory process and the development of chronic kidney disease (CKD) as a consequence of the deposition of immunocomplexes in the kidney tissue (Berrahal et al., 1996; Solano-Gallego et al., 2011; Greene, 2012; Godoy et al., 2017).

One of the main consequences of CKD is the development of secondary renal hyperparathyroidism, influenced by complex interactions between ionized calcium (Ca_i), phosphorus, vitamin D metabolites, parathormones (PTH), and the fibroblast growth factor 23 (FGF-23) (Rudinsky et al., 2018; Parker et al., 2017).

Inflammatory processes, alterations in iron and ferritin metabolism, and the decrease in renal function can influence the hormones involved in the regulation of circulating phosphorus and calcium, resulting in the increase of blood PTH and excretion of urinary phosphorus in animals with CKD (Fliser et al., 2007; David et al., 2016; Kanbay et al., 2017). In animals with VL, the inflammatory process is exaggerated due to the exacerbated humoral response, triggering several clinical and laboratory signs, such as eye changes, dermatitis, pododermatitis, polyarthritis, glomerulonephritis, and anemia of inflammation (Solano-Gallego et al., 2011). Therefore, changes in the urinary fractional excretion of phosphorus (uFEP) are expected in early phases of CKD in dogs with VL (Gutierrez et al., 2005; Wolf, 2010; Golf, 2007; Riella, 2014).

Therefore, this study aimed to determine the concentration of serum phosphorus, ionized calcium (Ca_i), uFEP, and PTH in azotemic and non-azotemic dogs with VL and evaluate the utility of the uFEP in the early detection of secondary renal hyperparathyroidism.

MATERIAL AND METHODS

This study was approved by the Ethics Commission in Animal Experimentation (CEUA/UFMS), under the protocol number 897/2017.

Twenty-four dogs of both sexes, with age ranging from nine months to ten years and diagnosis of leishmaniasis, were included in the study. These animals were treated at the Veterinary Hospital of the Federal University of Mato Grosso do Sul. Seven clinically-healthy animals formed the control group.

The inclusion criteria were: (1) diagnosis of VL confirmed through positive parasitological lymph node examination or immunochromatographic test (DPP® test for Canine Leishmaniasis, Brazil); (2) no previous treatment for the disease; (3) proteinuria above 0.5 in the examination of the urinary protein/creatinine ratio (UP/C). Pregnant or lactating animals, under use of calcium or phosphorus supplementation, and with clinical alterations or infirmities not related to leishmaniasis were excluded from the study.

Blood samples were collected in tubes with EDTA (ethylenediaminetetraacetic acid) and in tubes containing clot activator. Phosphorus, Ca_i, and creatinine measurements were performed with serum aliquots, and the PTH concentration was measured in the plasma. The urine was obtained by cystocentesis or urinary catheterization, and the samples were subjected to urinalysis, UP/C determination, and analysis of the concentrations of creatinine and phosphorus. The uFEP was calculated based on the formula proposed by DiBartola et al. (1980), as follows:

$$\frac{(Fósforo\ urinário/Fósforo\ sérico)}{(Creatinina\ urinária/Creatinina\ sérica)} \times 100$$

Urinalysis was performed immediately after the collection. For the biochemical and hormonal dosages, the serum and plasma samples were separated in up to 30 minutes after collection and frozen at -20 °C until the processing.

The biochemical examinations and the measurement of creatinine and urinary phosphorus were performed in a COBAS C111® automatic analyzer (Roche®, USA), and the urinary protein was measured in a semiautomatic BIO-200® analyzer (Bioplus®, Brazil) with a commercial kit. The PTH evaluation was performed with a COBAS e411® analyzer (Roche®, USA) with specific human kits, following the already-validated methodology for dogs, by other authors (Cortadellas et al., 2010; Harjes et al., 2017). The Ca_i was measured with a COBAS C221® blood gas analyzer (Roche®, USA).

The positive animals for leishmaniasis were classified into two groups: a group with non-azotemic CKD (GNA) and a group with azotemic CKD (GA). The criterion for the establishment of CKD was the presence of UP/C values above 0.5, whereas azotemia was defined by serum creatinine above 1.4 mg/dL, according to the recommendations of the International Renal Interest Society (IRIS, 2019). The control group (GC) was composed of clinically-healthy animals with a negative immunochromatographic test for VL (DPP® test for Canine Leishmaniasis, Brazil).

The serum analytes, PTH and uFEP, were compared between groups using the Kruskal-Wallis test with a 0.05 significance level. The Mann-Whitney test was employed as a *post hoc* test for the two-by-two comparison. In order to control the type-I error, the significance level used in the Mann-Whitney test was 0.016 (0.05/3 – number of two-by-two comparisons performed). All statistical tests were calculated according to Zar (1999).

RESULTS AND DISCUSSION

The GNA group was composed of 17 animals, and the GA and GC groups were represented by 7 animals each. Table 1 presents the comparisons between groups for the serum analytes, plasma PTH, and percentage of the uFEP.

Table 1. Median, amplitude, and statistical difference between negative dogs for VL and azotemic and non-azotemic positive dogs of different serum analytes, plasma parathormone, and uFEP.

	GC (med. - amp.) n=7	**GNA (med. - amp.) n=17**	**GA (med. - amp.) n=7**
Creatinine (mg/dL)	0.8-0.8^{A}	0.6-1.2^{A}	3.3-10.3^{B}
PTH (pg/mL)	7.71-68.98^{A}	274.6-329.5^{B}	402.4-342.0^{B}
Phosphorus (mg/dL)	4.04-1.58^{A}	4.48-4.95^{A}	12.0-31.9^{B}
Ca_i (mmol/L)	1.19–0.17^{A}	1.22-0.63^{A}	1.12–0.78^{A}
uFEP (%)	19.93-16.43$^{A, B}$	14.67-26.66^{A}	36.46-45.74 ^{B}

GC- control group, GNA- non-azotemic group, GA- azotemic group; med.- median; amp.- amplitude; PTH- parathormone; Ca_i – ionized calcium, uFEP – urinary fractional excretion of phosphorus. Difference between letters in the same row shows a statistical difference in the Mann- Whitney test ($p< 0.016$), significant after the Kruskal – Wallis test ($p<0.05$).

No statistical difference was observed in the serum creatinine values between the GNA and the GC groups ($p= 0.23$), although a decrease was noted in the value of the median of serum creatinine in the GNA (Table 1). These results were also observed by Cortadellas et al. (2008). The numerical decrease of serum creatinine can be explained by the hyperfiltration that occurs in the early stages of CKD, as a compensatory mechanism, and predicts the development of nephropathies, with deleterious effects on the long-term survival of the remaining nephrons (Chew et al., 2011). Another contributing factor is the presence of cachexia, common in dogs with VL, which results in lower levels of serum creatinine (Chew et al., 2011; Solano-Galego et al., 2011).

High creatinine values in the GA were expected since the classification of groups was performed based on the presence of serum creatinine above 1.4 mg/dL. However, it is necessary to highlight the presence of a high variation in the amplitude of the analyte (10.3 mg/dL). This variation is noted due to the presence of azotemic patients in several CKD stages (3 in stage 2, 1 in stage 3, and 3 in stage 4 – classification according to IRIS, 2019).

This occurs since the animals with VL develop CKD by glomerular injury due to the accumulation of immunocomplexes in the endothelial and subendothelial regions of glomerular arteries. The accumulation of these antigen-antibody complexes attracts inflammatory cells, lymphocytes, neutrophils, and macrophages, which contribute to the inflammatory process with the release of autacoids. CKD is a progressive and irreversible disease since with the lasting inflammatory process in the glomerulus the fibrous tissue begins to fill the place, with later calcification of the already unusable nephrons. As the replacement of nephrons progresses, the glomerular filtration rate declines. As a consequence, substances with excretion exclusively via urine accumulate in the organism, resulting in increasingly severe azotemia (Chew et al., 2011; Solano-Galego et al., 2011; Greene, 2012; Godoy et al., 2017).

Harjes et al. (2017) and Cortadellas et al. (2008) found high PTH concentrations in azotemic dogs when compared to healthy animals. Similar results were observed in the present study, although the GNA also presented a statistical difference with the GC (Table 1). The PTH increases in response to the decrease of Ca_i and/or the increase of serum phosphorus and/or the decrease of calcitriol. Calcitriol, in its turn, decreases due to the increase of FGF-23 and the loss of functional renal mass (Galvão et al., 2013; Riella, 2014).

David et al. (2016) observed that inflammatory processes can increase the plasma levels of FGF-23, mainly affecting individuals with CKD that possess less renal tissue responsible for the catabolism of this hormone (Kanbay, 2017). In its turn, the FGF-23 stimulates PTH production and inhibits the production of calcitriol. The last one performs an important role in the negative feedback of the PTH (Cunningham et al, 2011; Galvão et al, 2013). Thus, individuals with CKD and inflammatory processes, such as in the VL, possess significant alterations in the levels of PTH even in the early stages of the disease.

Brow et al. (1989) found a lower number of calcitriol receptors in the parathyroid glands of azotemic dogs, whereas Gerber et al. (2003) and Cortadellas et al. (2008) observed a decrease in the calcitriol levels in azotemic dogs. These data demonstrate that animals with CKD present a marked difficulty in controlling the plasma concentrations of PTH since calcitriol performs a negative feedback control of this hormone. Furthermore, the decrease in the calcitriol levels is related to the increase of the inflammatory process (Yin and Agrawal, 2014).

Thus, high PTH levels in the GNA can be justified by the chronic inflammatory process, evidenced in this group of animals by the presence of clinical signs (16/17) and non-regenerative anemia (16/17), increasing the FGF-23, which possesses a positive feedback role with the PTH and negative feedback with calcitriol (Cunningham et al., 2011; Galvão et al., 2013; Riella, 2014). The decrease in calcitriol contributes to the inflammatory response of the patient, creating an inflammation cycle,

the increase of FGF-23, decrease of calcitriol, and increase of PTH.

The Ca_i was not statistically different between groups (Table 1). This finding resembles that verified by other authors, who observed no significant differences in the total calcium and Ca_i between azotemic and non-azotemic animals, with or without VL (Cortadellas et al., 2009; Harjes et al., 2017). However, Cortadellas et al. (2008) verified lower concentrations of Ca_i in patients with advanced spontaneous CKD. Although no statistical difference was observed in the present work, the GA group presented the lowest median of Ca_i (Table 1), with 28% of the animals below 1 mmol/L. Patients with mild and moderate CKD did not present alterations in the plasma concentrations of Ca_i due to the same compensatory mechanisms that maintain phosphorus homeostasis (Galvão et al., 2013; Riella, 2014). However, there is a great alteration in the behavior of calcium among patients in later stages of CKD due to the individual variation in the fixation rate of the mineral with other molecules (Chew et al., 2011), unlike phosphorus.

The values of serum phosphorus in the GNA group presented no statistical difference in relation to those of the GC (p= 0.57) (Table 1). Patients in the early stages of CKD often presented normal serum concentrations of phosphorus due to the corrective effect of regulating hormones, demonstrating hyperphosphatemia when approximately 85% of the renal function was compromised (Levin et al. 2007; Chew et al., 2011). However, two patients of the GNA group (8.5%) presented hyperphosphatemia (serum phosphorus >5.5 mg/dL). Cortadellas et al. (2009) already reported the existence of hyperphosphatemia in non-azotemic animals with VL (12%), demonstrating that alterations in this analyte may occur still in the early stages of CKD when VL is present. These findings reinforce the need to quantify the serum phosphorus in animals with VL even without azotemia, and if necessary, to begin treatment with an adequate diet and phosphorus chelators even in the early stages of the disease.

As for the GA, a marked hyperphosphatemia was observed. This data corroborates the results verified by Cortadellas et al. (2009) and Martoreli et al. (2017), who associated hyperphosphatemia with late stages of CKD. In the present study, 42% of the GA animals were in the final stage of the CKD, with less than 15% of preserved renal function (Chew, 2011; IRIS, 2019).

There was a statistical difference in the urinary fractional excretion of phosphorus between the GNA and the GA groups (p= 0.014) (Table 1). The increase in the uFEP was significant in the GA, showing to be a compensatory mechanism in the attempt to maintain the serum levels of phosphorus within the normality, although this mechanism becomes less efficient with the progression of the disease and the decrease in the functional renal mass (Martoreli et al., 2017). No statistical difference was observed between the GA and the GC (p= 0.11) (Table 1), which may be associated with the reduced sample size of both groups and the high amplitude in the GA, hindering the visualization of statistical differences.

Although the animals in the GNA presented high PTH values, no statistical difference was observed in the uFEP between the GC and the GNA groups (p=0.09) (Table 1). Numerically, a reduction was observed in the median of the uFEP of the GNA. This may occur as a consequence of the hyperfiltration in the remaining functional nephrons. This hyperfiltration reduces the values of serum creatinine and increases those of urinary creatinine. This results in a larger divider in the calculation of the uFEP, which leads to a lower calculated value of the uFEP. However, in this case, a higher phosphorus excretion was expected since the PTH and the FGF-23 interfere with the tubular phosphorus transporters that reabsorb this element from the ultrafiltered fraction (Riella, 2014). Therefore, other non-evaluated factors in this study may intervene in the uFEP in dogs with VL.

New studies focusing on the metabolism of calcitriol, FGF-23, PTH, and inflammatory markers must be performed in order to find therapeutic targets that can stop the development of secondary renal hyperparathyroidism in dogs with VL.

CONCLUSIONS

Dogs with VL possess high levels of PTH and hyperphosphatemia, even in the absence of azotemia. This reinforces the importance of quantifying phosphorus and PTH in these patients and, if necessary, to perform a therapeutic intervention even in the early stages of the disease. The uFEP showed to be a later marker of CKD and secondary hyperparathyroidism in dogs with VL. New studies aiming at the metabolism of phosphorus-regulating hormones are required in order to better understand the progression of chronic kidney disease in dogs with VL.

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