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Relation between anaemia and bone marrow features and serum erythropoietin in dogs with chronic kidney disease[1]

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ABSTRACT.- Torres M.M., Cruz F.A.C.S., Silva E.P., Poletto D., Cayuela M.A.A., Mendonça A.J., Almeida A.B.P.F. & Sousa V.R.F. 2017. **Relation between anaemia and bone marrow features and serum erythropoietin in dogs with chronic kidney disease**. *Pesquisa Veterinária Brasileira 37(6):598-602.* Departamento de Clínica Médica Veterinária, Faculdade de Medicina Veterinária, Universidade Federal de Mato Grosso, Av. Fernando Corrêa da Costa 2367, Boa Esperança, Cuiabá, MT 78060-900, Brazil. E-mail: marys_torres@hotmail.com

Chronic anaemia is one of the most severe complications of chronic kidney disease, contributing to morbidity and mortality caused by the disease; therefore, bone marrow cytological evaluation is needed to monitor the progression of anaemia. This study aimed to correlate the anaemia in dogs at different stages of chronic kidney disease with their serum biochemistry, myelogram results and serum erythropoietin findings. Sixty-three dogs were grouped according to International Renal Interest Society (IRIS) classification in stages 1, 2, 3 and 4. Haematologic, serum and urinary biochemistry and serum erythropoietin were performed for comparison with the findings of bone marrow cytology obtained by aspiration of the manubrium. Cytological findings for erythroid hypoplasia were described in 93.65% of dogs, and the anaemia was observed in 84.1% of them. The haematological findings were correlated with azotaemia ($p<0.05$). It was concluded that the erythroid hypoplasia has correlation with persistent anaemia in dogs at all stages of chronic kidney disease, with iron deficiency in dogs in the early stages and with peripheral destruction of erythrocytes caused by azotaemia.

INDEX TERMS: Anaemia, bone marrow, serum erythropoietin, dogs, chronic kidney disease, cytology, erythrocytes, iron metabolism, myelogram, renal.

RESUMO.- [**Relação entre anemia e achados de medula óssea e eritropoetina sérica em cães com doença renal crônica.**] A anemia crônica é umas das complicações mais graves da doença renal crônica, contribuindo para a morbidade e mortalidade causada pela doença; Portanto, a avaliação citológica da medula óssea é necessária para monitorar a progressão da anemia. Assim, esse estudo objetivou correlacionar a anemia em cães em diferentes estágios da doença renal crônica aos achados de bioquímica sérica, mielograma e concentração sérica de eritropoietina. Sessenta e três cães foram agrupados de acordo com a classificação da International Renal Interest Society (IRIS) em estágios 1, 2, 3 e 4. Foram realizadas análises hematológicas, bioquímicas séricas e urinárias, e dosagem sérica de eritropoetina para comparação com os achados medulares obtidos por citologia aspirativa do manúbrio. Os achados citológicos de hipoplasia eritróide foram descritos em 93,65% dos cães, e a anemia foi observada em 84,1% dos cães. Os resultados hematológicos foram correlacionados com azotemia ($p<0,05$). Concluiu-se que a hipoplasia eritróide teve associação com a anemia persistente em cães em todas as fases de doença renal crônica, com deficiência de ferro em cães em fases iniciais e com a destruição periférica dos eritrócitos causada pela azotemia.

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TERMOS DE INDEXAÇÃO: Anemia, medula óssea, eritropoetina sérica, caninos, doença renal crônica, citologia, eritrócitos, metabolismo do ferro, mielograma, renal.

INTRODUCTION

Chronic anaemia, usually of the normocytic normochromic nonregenerative type, is a common and invariable complication of chronic kidney disease (CKD), resulting from decreased erythrocyte half-life, iron and folic acid deficiency, the action of uremic toxins and, especially, erythropoietin deficiency (Waki et al. 2010, Macdougal 2011, Polzin 2011, Zadrazil & Horak 2014).

In humans, CKD anaemia is correlated with cardiovascular disturbances and other comorbidities (Palmer et al. 2010). Similarly, in dogs, the production of erythropoietin is markedly reduced in CKD, leading to anaemia that is difficult to treat. With any of the several treatments proposed to treat anaemia associated with CKD, regardless of the adopted therapy, the mensuration of erythropoietin serum concentration as a part of therapeutic monitoring is fundamental (Giampaoli et al. 2003).

The cytological evaluation of bone marrow allows for the analysis of a patient's response to the progression of the anaemia, acting as another important tool for monitoring the development of the CKD. The anaemia becomes more evident in advanced stages of CKD, compromising the quality of life and contributing to the high morbidity and mortality of the dogs (IRIS 2013, Zadrazil & Horak 2014, Kogika et al. 2015).

Therefore, this study aimed to correlate the anaemia in dogs at different stages of CKD with their serum biochemistry, myelogram results and erythropoietin serum concentration.

MATERIALS AND METHODS

Ethics statement. This study was approved and conducted in accordance with the recommendations of the Animal Care and Use Ethics Committee of the University of Mato Grosso (protocol # 23108.043331/12-9 CEUA).

Animals. The prospective study included dogs of different breeds and both sexes with CKD. As inclusion criteria, were selected dogs with or without anaemia, presenting azotaemia and hyposthenuria and which have not undergone to any treatment for this condition, as well as for high blood pressure. They were grouped according to the International Renal Interest Society (IRIS 2013) criteria, based on the creatinine serum concentration in: stage 1 (<1.4mg/dl), stage 2 (1.4-2.0mg/dl), stage 3 (2.1-5mg/dl) and stage 4 (≥5mg/dl). The substaging was determined through the assessment of the systolic blood pressure, measured in triplicate in all dogs by oscillometry (Pet Map®, Rio de Janeiro, Brazil), and of the presence of proteinuria by urine protein-to-creatinine (UPC) ratio.

Blood, urine and bone marrow samples were obtained from dogs for the following diagnostic tests: Complete blood count (CBC), serum and urinary biochemistry, urinalysis, dosage of serum erythropoietin and myelogram.

Another group comprising healthy dogs of different breeds and both sexes was used to obtain reference parameters, with those dogs presenting haematological, biochemical and urinary disorders being excluded.

Complete blood count and biochemistry. For the CBC, the haematocrit, haemoglobin, red blood count, mean corpuscular haemoglobin concentration (MCHC), mean corpuscular volume (MCV) and platelet count were assessed with an automatic analyser (Sysmex pocH-100iV, São Paulo, Brazil).

The serum biochemistry were assessed with a semiautomatic analyser (Celm SB-190®, São Paulo, Brazil) and commercial kits (Gold Analisa®, Belo Horizonte, Brazil) to determine levels of urea, creatinine, iron, total iron binding capacity (TIBC), calcium and phosphorus. The UPC ratio was obtained by calculating the ratio of the amount of urinary protein and urinary creatinine determined with a semiautomatic analyser (Celm SB-190®, São Paulo, Brazil) and commercial kits (Gold Analisa®, Belo Horizonte, Brazil), according to Castro et al. (2009).

Myelogram. Marrow aspirates were obtained from the manubrium of each dog, with EDTA (3%) being used as the anticoagulant. Then, the smears containing bone spicules were submitted to Romanowsky staining.

The evaluation was based on cellular identification according to the morpho-tinctorial features of the cells. A total of 500 cells were counted and the percentages of erythroid and myeloid cells and the myeloid/erythroid ratio were calculated, and the cytological findings were described. The cellularity of bone marrow was estimated by examining the proportion of cells versus fat, considering as a hypocellular marrow when the particles were composed of more than 75% of fat (Harvey 2001).

Reference values were those previously described (Harvey 2001, Weiss & Smith 2002).

Serum erythropoietin. The serum erythropoietin in dogs was determined with an ELISA sandwich approach using a canine-specific commercial kit (Cloud-Clone Corp.®, Houston, TX, USA).

Statistical analysis. The normality of the data was determined using the Kolmogorov-Smirnov test. An analysis of variance was employed for comparisons among the CKD stages, and the Pearson's correlation coefficient was applied to the data on the bone marrow findings. The statistical analyses were performed with GraphPad Prism 4 software.

RESULTS

Sixty-three dogs, including 29 females and 34 males, with a mean age of 6.7 years (ranging from one to 16 years) and of varied breeds, composed the CKD group. Nine healthy dogs of different breeds, six males and three females with an average age of three years (one to five years), comprised the control group.

Among the CKD dogs, 11 were classified as being in stage 1, eight were classified as being in stage 2, 22 were classified as being in stage 3 and 22 were classified as being in stage 4. Sub-staging was determined by systolic blood pressure and by the UPC ratio, with all dogs showing proteinuria (>0.5) and hyposthenuria, along with a specific urinary gravity ranging from 1008 to 1018.

A high systolic blood pressure (≥180 mmHg) was observed in 34% of the dogs, with a predominance occurring in stage 4 in comparison with the other stages (p=0.02).

Clinical signs observed in CKD dogs ranged in severity from occasional vomiting (47.6%) and inappetence (93.6%) to severe anorexia (19%), frequent vomiting (52.4%), tongue necrosis (1.6%) and pale mucosa (62.26%).

Regarding the erythrogram, all the assessed parameters, except MCV and MCHC, declined as the CKD progressed, which characterizes the normocytic normochromic

anaemia observed in 84.1% of the dogs. Of these, 37.5% had severe anaemia (haematocrit ≤ 20%) with reticulocytosis indexes ranging from zero to 13.2% (mean 2.7%), and 11.1% presented normocytic hypochromic anaemia.

The red blood count, haematocrit and haemoglobin levels showed statistically significant differences only compared to those of the control dogs (p≤ 0.05). Thrombocytopenia occurred in 39.6% of the anaemic dogs; however, there was no significant difference (p=0.81) among the CKD stages.

No significant difference (p= 0.37) between stages was observed for the myeloid/erythroid ratio, as determined from the myelogram. The marrow findings (Figure 1) of the CKD dogs are described in Table 1.

In the serum biochemistry, the calcium, phosphorus, total iron binding capacity and iron concentrations did not show significant differences among the CKD stages, except for the serum phosphorus, which showed a significant difference in dogs at stage 4 in comparison to the control group (Table 2).

The concentration of erythropoietin was determined in 44 of the 63 dogs in the study. The values obtained were not correlated with the bone marrow or erythrogram findings.

Among the dogs diagnosed with erythroid hypoplasia,

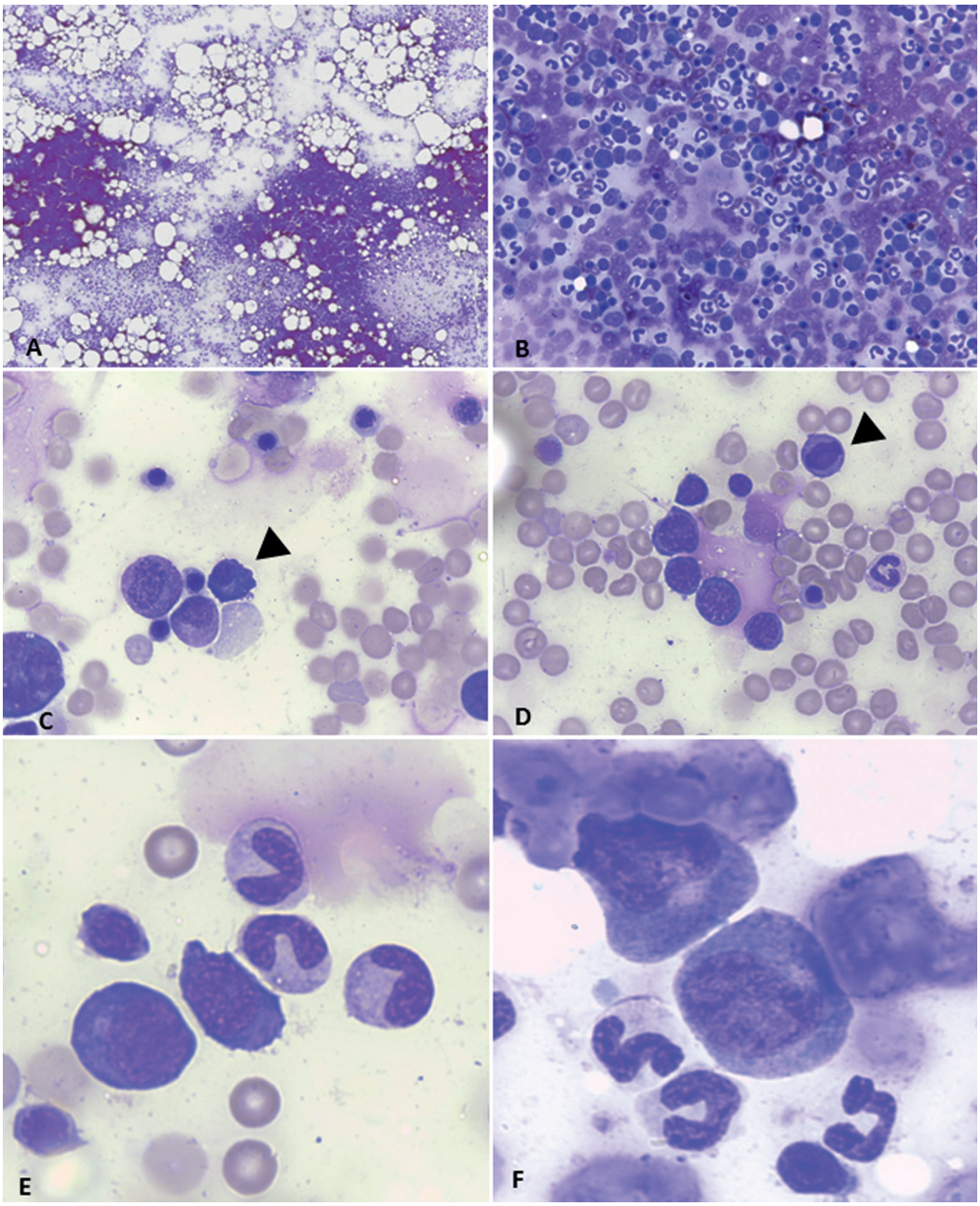

Fig.1. Bone marrow aspirate in a dog with chronic kidney disease in stage 4. Romanowsky stain. (**A**) The normal marrow cellularity. Obj.10x. (**B**) Myeloid cells in higher proportion than erythroid, presence of several plasmocytes. Obj.40x. (**C**) Plasmocyte (arrow head). Obj.100x. (**D**) Dyserythropoiesis (arrow head) and prorubricytes. Obj.100x. (**E**) Two band cells and metamyelocyte. Obj.100x. (**F**) Myeloblasts and band cells. Obj.100x.

19 presented with haematocrit ≤ 20%, and nine of them did not present reticulocytosis. Additionally, whereas megakaryocytic hyperplasia was present in five dogs; two of these also presented with thrombocytopenia.

Myelodysplastic syndrome was described in two dogs based on the presence of dysmyelopoiesis and rubriblasts observed in 7 to 18% of the nucleated cells.

Serum iron levels were correlated with erythroid precursors, with a significant correlation with the myeloid/erythroid ratio (r= -0.95) and the percentage of rubriblasts (r= -0.89), rubricytes (r= 0.89) and metarubricytes (r= 0.91) being observed in stage 2 dogs, whereas in stage 4 dogs, a correlation was observed only with the myeloid/erythroid ratio (r= 0.66).

The haematocrit of all dogs was correlated with the serum biochemistry, serum levels of erythropoietin and the myeloid/erythroid ratio, as shown in Table 3.

DISCUSSION

According to the IRIS (2013) classification for CKD, the initial stages of 1 and 2 tend to be subclinical, explaining why most of the dogs in this study belong to stages 3 and 4. These dogs were also classified as proteinurics and hypertensives in all CKD stages, with systolic blood pressure higher than 180 mmHg observed predominantly in stage 4. Proteinuric and/or hypertensive patients can present clinical manifestations in the early stages of CKD; therefore, the classification and sub-staging proposed by IRIS (2013) allows the assessment of renal damage and associated complications, which can serve as a prognostic factor (Waki et al. 2010, Polzin 2011).

Although there is no breed or age predilection (Polzin 2011), the morbidity and mortality occur predominately in elderly patients (Lees 2004), as demonstrated by the dogs in this study.

In humans with kidney disease, a strong correlation exists between the degree of anaemia and a risk of morbidity and mortality from cardiovascular diseases (Locatelli et al. 2003). Although this correlation was not significant in our study, the fact that 34% of the CKD dogs were hypertensive (systolic blood pressure ≥ 180 mmHg), exhibited increased azotaemia, and were in the advanced stages classifies these dogs as critically ill.

In CKD dogs, the azotaemia, acting mainly on the haematocrit, proved to be a major factor in the persistence of anaemia ($p<0.05$) due to the progressive deterioration in renal function, culminating with decreased haemoglobin concentration, which becomes evident when the glomerular filtration rate declines (Macdougall 2011).

The effects of uremia on the oxidative stress in erythrocytes in anaemic dogs with CKD was previously studied, and although no correlation was demonstrated, anaemia is associated with reduced erythrocyte lifespan (Kogika et al. 2015). Although the oxidative stress assessment has not been performed in our study, the uremia described could be one of the factors involved in chronic anaemia and a possible cause for the described peripheral alterations due to the significant correlation among the haematocrit and azotaemia observed in our study.

Furthermore, hyperphosphatemia contributes to this

Table 1. Cytologic features of the bone marrow in dogs with chronic kidney disease

Parameters	Nº	%
Normal cellularity	38	60.31
Myeloid hyperplasia	20	31.74
Megakaryocytic hyperplasia	05	7.93
Erythroid hypoplasia	59	93.65
Myeloid hypoplasia	04	6.35
Lymphocytosis	06	9.52
Plasmocytic hyperplasia	07	11.11
Dysgranulopoesis	05	7.93
Myelodysplastic syndrome	02	3.17

Table 2. Summary of the serum concentration statistics for calcium (Ca), phosphorus (P), iron, iron binding capacity (TIBC) and erythropoietin (EPO) in the diseased and control groups

Parameters	Stage 1 n= 11	Stage 2 n= 08	Stage 3 n= 22	Stage 4 n= 22	Control n= 09
Ca (mg/dl)					
Mean	11.44	10.45	11.18	11.78	12.52
Median	11.57	10.53	11.50	11.40	12.54
Standard deviation	1.66	0.96	3.33	2.38	0.51
p-value*	0.7				
P (mg/dl)					
Mean	2.08	1.75	2.97	3.58a	1.1a
Median	1.45	1.60	2.20	3.20	1.1
Standard deviation	1.75	0.88	1.95	1.95	0.12
p-value*	0.04				
Iron (µg/dl)					
Mean	195.8	145.8	146.4	109.0	233.8
Median	193.0	115.0	128.5	83.00	230.0
Standard deviation	89.93	85.48	108.1	112.3	113.0
p-value*	0.07				
TIBC (µg/dl)					
Mean	565.6	599.8	556.7	572.1	478.8
Median	584.0	590.5	487.0	528.0	506.0
Standard deviation	92.19	65.22	204.7	200.2	139.8
p-value*	0.83				
EPO**					
Mean	1421	-	1474	1461	1499
Median	1469	-	1480	1484	1501
Standard deviation	112.1	-	51.61	80.23	18.09
p- value*	0.27				

*Analysis of variance (Ca, P, Iron, TIBC). EPO = control vs stages). a Means followed by the same letter in the same row differ at the 5% level of significance. ** Evaluated in 44 dogs (39 CKD.

Table 3. Spearman's rank correlation between the haematocrit and the myeloid/erythroid ratio (MER), serum iron, total iron binding capacity (TIBC), calcium, phosphorus, urea, creatinine and erythropoietin (EPO) in diseased dogs

Variables	r	p-value
Haematocrit and MER	0.13	0.49
Haematocrit and Iron	0.10	0.39
Haematocrit and TIBC	-0.13	0.35
Haematocrit and Calcium	-0.02	0.86
Haematocrit and Phosphorus	0.09	0.45
Haematocrit and Urea	-0.33	0.003*
Haematocrit and Creatinine	-0.27	0.01*
Haematocrit and EPO	0.0006	0.99

* Significant difference.

process, mainly in the advanced stages of CKD, as observed in the dogs in stage 4. Serum phosphorus level elevation, in association with normal calcium levels, were also reported in a majority of dogs in a previous study (Kogika et al. 2006). In CKD, such an imbalance can be observed from the earliest stages of the disease (Cortadellas et al. 2010) and may result in renal secondary hyperparathyroidism, which implies a lower survival for these dogs (Geddes et al. 2013).

Regarding the serum iron concentrations, although no differences were observed among the stages, the effect of these concentrations on erythroid cells, primarily in stages 2 and 4, show that iron acts on mature cell lines in one way and, curiously, in an inverse way on immature cell lines. This mild anaemia may be the result of inflammatory disease and, additionally, it reduces the erythrocyte lifespan and its responsiveness to the anaemia due to low erythropoietin synthesis and iron deficiency, thereby culminating in limited erythropoiesis (Almeida et al. 2009).

Iron serum dosage is one of the confirmation methods of this deficiency when the haematological findings suggest hypochromic microcytic anaemia, this diagnosis can be affirmed based on our results which prove that in CKD, the occurrence of hyposideremia has lower relation with anaemia. Furthermore, microcytosis is a good indicator of iron deficiency only when MCV values are extremely low, and this situation, which was not reported in our study, is related to blood loss (Paltrinieri et al. 2010).

As previously shown, differences between the means of the serum levels of erythropoietin and iron in the different CKD stages were not observed and did not correlate with the myelogram and CBC findings; however, the normal values of the myeloid/ erythroid ratio in anaemic dogs and the low reticulocytosis indexes in 47.3% of the dogs with erythroid hypoplasia indicate erythropoiesis suppression. Macdougall (2011) suggests that in addition to the observed decrease in erythropoietin production, decreased erythropoietin activity on bone marrow may also occur. This mechanism could explain our erythropoietin findings.

The most frequent anaemia in CKD is normocytic normochromic anaemia (Macdougall 2011), as reported in 84.1% of the dogs in this study. This form of anaemia is described as frequent and recurrent (Weiss 2006, Raskin & Messik 2012).

CONCLUSIONS

The erythroid hypoplasia observed in CKD dogs is preponderant in the persistence of anaemia from the early stages of this disease on.

The erythroid hypoplasia was associated with iron deficiency in the initial stages and with peripheral erythrocyte destruction caused by progression of the azotaemia.

Cytological examination of the bone marrow enabled to determinate the erythropoiesis suppression occurring in the CKD dogs, primarily against mature erythroid progenitors, which emphasizes the usefulness of this method as a prognostic tool in anaemic CKD patients.

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