# Polymorphonuclear function in naturally occurring renal failure in dogs

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**ABSTRACT**: Chronic renal failure causes immunosuppression in people and is thought to be one of the causes of non-infectious secondary immunosuppression in dogs. The purpose of this study was to evaluate changes in counts and activity of polymorphonuclears in dogs with chronic renal failure in various stages. Haematological, biochemical examinations and examination of non-specific immune response cells (total and differential leukocyte counts, phagocytosis of methacrylate particles, chemiluminescence test, and level of lysozyme) were performed in blood samples obtained from these dogs. Neutrophilia, lymphopoenia and a decreased number of eosinophils in comparison with healthy control were the main findings in groups with clinical signs. We found the statistically highly significant elevation of lysozyme level; it was in a strong positive correlation with the level of urea, creatinine and phosphorus. We did not find any statistically significant changes in phagocytosis process and other serological factors. In conclusion, despite the reports from human medicine, chronic renal failure in dogs does not alter phagocytosis. From this aspect, the elevation of lysozyme level is the main effect of uraemia.

**Keywords**: leukocytes; uraemia; phagocytosis

Chronic renal failure (CRF) is a common problem of ageing dogs and cats. Renal failure is defined as a loss of three-quarters of functioning nephrons and it is associated with various clinical signs. The most common is polyuria and polydipsia due to the loss of concentrating ability. Gastrointestinal complications (inappetence, anorexia, vomiting, diarrhoea, weight loss) are very common and they are usually the first signs why owners come to veterinary practice. Neurological abnormalities associated with CRF are very common as well. They include dullness, lethargy, tremors, seizures, stupor and coma. The severity of these clinical signs may vary. The patients can be presented at various stages of the disease, ranging from subclinical (detected by laboratory tests only – the presence of azotaemia and inadequate urinary concentrating ability), mild (vomiting, weight loss, mild neurological signs) to severe azotaemia (end stage, when the homeostasis is so disturbed that it is incompatible with life). Chronic renal failure is associated with many changes in laboratory test. The presence of azotaemia and hyperphosphataemia is typical, common finding is anaemia (nonregenerative, normochromic and normocytic).

Chronic renal failure causes secondary immunosuppression. This is well documented in men, where infections are still a major cause of morbidity and mortality in end-stage renal disease patients. There are many reports of neutrophil dysfunction and increased risk of infection in these patients (Vanholder and Ringoir, 1992; Vanholder et al., 1993; Sharma et al., 2000). The functional impairment described in uremic neutrophils is there-

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fore mainly a result of their reduced ability to kill microorganisms intracellularly and is believed to increase the susceptibility to infections (Anding et al., 2003). The mechanism responsible for reduced neutrophil functions is not well understood, although a number of partly interdependent factors have been proposed, including iron overload, zinc deficiency, increased intracellular calcium, anaemia, malnutrition and dialysis therapy per se (Lewis and Parameter VanEpps, 1987). Furthermore a number of uremic toxins that affect the neutrophil function have been identified and characterized (Cohen et al., 1997).

The polymorphonuclear activity has not yet been evaluated in dogs with chronic renal failure. The purpose of this study is to determine possible changes in this part of immune response of these patients.

## MATERIAL AND METHODS

### Animals

Forty-five dogs with the diagnosis of chronic renal failure were assessed in the study. All these dogs were patients of the Clinic of Dogs and Cats Diseases, Faculty of Veterinary Medicine, University of Veterinary and Pharmaceutical Sciences, Brno, Czech Republic and were presented to clinic from May 2005 to June 2007. Dogs were diagnosed with CRF by the presence of persistent azotaemia (the serum creatinine level above the laboratory reference range of 125 μmol/l) in conjunction with poor urinary concentrating ability. Haematological examination, standard biochemistry profile and urinanalysis were performed in all dogs, ultrasonographic examination of kidneys was performed in 36 dogs. In cases where we were not able to differentiate acute and chronic renal failure, histopathological examination of renal tissue was performed. Animals with signs of significant extrarenal disease or prerenal/postrenal azotaemia at the time of initial diagnosis were excluded from the study. Animals with CRF and second disease which can cause secondary immunosuppression (e.g. hyperadrenocorticism, hypoadrenocorticism, diabetes mellitus, neoplasia, pyometra) or patients that had received corticosteroids were excluded as well.

There were twenty-four females (five of them neutered) and twenty-one males (one of them neutered) of various breeds. Average age was 9.85 years (from five months to sixteen years).

Dogs were divided into three groups according to the extent of clinical signs at the time of diagnosis. Dogs without clinical signs of CRF were in the first group. Azotaemia was found by routine serum biochemistry examination and diagnosis of chronic renal failure was proved by other findings (urinanalysis, ultrasonographic examination).The second group comprised dogs with clinical signs of uraemia (vomiting, inappetence, anorexia) with good response to therapy. In the third group there were dogs in the end stage of renal failure; these animals were not able to deal with changes of homeostasis. These dogs died or were euthanized shortly after diagnosis because of no response to therapy. A description of the groups is in Table 1.

Forty-five dogs were diagnosed with CRF according to history, clinical findings, haematological and biochemical examination and urinanalysis. The diagnosis was confirmed by ultrasonography and in some case by histopathological examination of renal tissue. More severe clinical signs occurred when the levels of creatinine and urea increased. In addition, it was in a strong correlation with diminishing number of red blood cells. Anaemia seen in these animals was typically non-regenerative. Main biochemical parameters (serum creatinine, urea and phosphorus) and red blood cells are shown in Table 2. Isostenuria was a typical finding and we found both active and passive urine sediment. In

Table 1. Characteristics of dogs, number in each group, average age (mean and standard deviation) and their clinical signs

| Parameter | Control dogs | Group 1 | Group 2 | Group 3 | Subgroup 3 (lymphopoenic) |
|---|---|---|---|---|---|
| Number | 15 | 10 | 15 | 20 | 10 |
| Sex (M/F) | 11/4 | 7/3 | 6/9 | 8/12 | 5/5 |
| Average age (years) | 9.6 ± 2.9 | 11.4 ± 2.5 | 9.2 ± 4.3 | 9.4 ± 4.3 | 9.9 ± 3.6 |
| Clinical signs | none without azotaemia | none with azotaemia | mild | severe end-stage CRF | severe end-stage lymphopoenia |

ultrasonography, the kidneys were mostly hyperechogenic. Diffuse chronic membranous glomerulonephritis was the most frequent result from histopathology.

**Control dogs**. Fifteen clinically normal dogs (eleven females, four of them neutered, four males, one of them neutered) of various breeds that came for vaccination to our clinic were included in the control group. The dogs were selected to be of similar age as CRF animals (average age 9.6 years). The dogs were considered to be healthy on the basis of history, physical examination findings and normal results of haematological and serum biochemistry analyses.

## Blood samples

Blood samples were taken by venipuncture from *v. jugularis* or *v. cephalica antebrachii*. The blood samples were collected into differential tubes: EDTA tubes for haematological examination, standard biochemistry tubes and heparinised tubes for immunological examination. All examinations were taken within 24 hours after collection.

## Immunological tests

**Total and differential leukocyte counts**. Total leukocyte counts were determined using the cell counter Digicell 500 (Contraves AG, Switzerland), differential leukocyte counts were enumerated from blood smears stained with May-Grunwald and Giemsa-Romanovski.

**Phagocytosis of methacrylate particles**. A slight modification of the phagocytosis test using methacrylate particles (MSHP) in whole blood (Vetvicka et al., 1982) was used to examine the ingestion activity of neutrophils and monocytes. Fifty µl of a suspension of MSHP particles (Artim, Prague) were mixed with 100 µl of blood in an Eppendorf type test tube and incubated at 37°C for one hour. Twenty µl of $Na_2EDTA$ ($10^{-4}$M) were added 5 min before the end of the incubation. Blood smears were prepared and stained according to Giemsa-Romanovski after the incubation. At least 200 cells were examined in each smear to determine the proportion of leukocyte types and the percentage of phagocytising cells, i.e. those in which at least three particles were engulfed.

The phagocytic index was calculated as the mean of the number of ingested particles in phagocytising cells (examined 100 cells at least).

## Chemiluminescence test

During the metabolic phase of phagocytosis phagocytic cells produce reactive oxygen radicals (hydrogen peroxide, superoxide, superoxide anion) that are very potent to kill bacteria and destroy ingested material. These radicals may provoke mild radiation in luminescent solutes (in this case luminol) that can be detected with a suitable photomultiplier. 100 µl of blood was mixed with 400 µl of medium for cell cultivation (MEM, pH 7.2), just before measurement 200 µl of luminol was added (spontaneous chemiluminescence) and into selected tubes 100 ml of rice starch was added (activated chemiluminescence). The samples were measured on a BioOrbit 1251 luminometer (BioOrbit, Finland) twenty minutes in two minutes intervals. The metabolic activity was evaluated according to two parameters: spontaneous activity and stimulation index, i.e. the ratio between spontaneous and stimulated activity.

Table 2. Characteristics of each group of dogs with chronic renal failure and group of healthy control (creatinine, urea and phosphorus levels expressed as a mean and standard deviation)

| Parameter | Control dogs | Group 1 | Group 2 | Group 3 | Subgroup 3 |
|---|---|---|---|---|---|
| Number | 15 | 10 | 15 | 20 | 10 |
| Clinical signs | none without azotaemia | none with azotaemia | mild | severe end-stage CRF | severe end-stage lymphopoenia |
| Creatinine (µmol/l) | 92.5 ± 16.9 | 194.8 ± 39.3 | 334.2 ± 136.4 | 939.8 ± 604.4 | 972.3 ± 658.2 |
| Urea (mmol/l) | 6.7 ± 2.4 | 20.0 ± 8.3 | 28.0 ± 13.8 | 61.6 ± 34.2 | 62.7 ± 36.5 |
| Phosphorus (mmol/l) | 1.4 ± 0.5 | 1.8 ± 0.5 | 2.5 ± 1.2 | 5.6 ± 1.8 | 5.0 ± 1.8 |

Table 3. Red blood cell counts and total and differential leukocyte counts in each group of dogs with chronic renal failure and healthy control (mean and standard deviation)

| Parameter | Control dogs | Group 1 | Group 2 | Group 3 | Subgroup 3 |
|---|---|---|---|---|---|
| Number | 15 | 10 | 15 | 20 | 10 |
| Clinical signs | none without azotaemia | none with azotaemia | mild | severe end-stage CRF | severe end-stage lymphopoenia |
| Red blood cells ($10^{12}$/l) | 6.9 ± 0.9 | 6.0 ± 1.6 | 5.6 ± 1.3 | 4.8 ± 1.8 | 5.59 ± 1.9 |
| Leukocytes ($10^6$/l) | 8 250 ± 1 818 | 12 023 ± 7 523 | 9 995 ± 2 312 | 10 311 ± 4 004 | 8 418 ± 2 548 |
| Neutrophils ($10^6$/l) | 5 506 ± 1 331 | 8 387 ± 6 904 | 7 822 ± 2 552** | 7 933 ± 3 495*** | 6 541 ± 2 863*** |
| Lymphocytes ($10^6$/l) | 2 075 ± 680 | 2 567 ± 1 271 | 1 573 ± 694** | 1 413 ± 1 211*** | 601 ± 237*** |
| Monocytes ($10^6$/l) | 303 ± 177 | 688 ± 554 | 404 ± 256 | 526 ± 309 | 524 ± 279* |
| Eosinophils ($10^6$/l) | 345 ± 282 | 381 ± 439 | 196 ± 244** | 125 ± 148*** | 124 ± 121*** |

*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ when compared with control dogs

## Lysozyme level

Lysozyme is an enzyme with antimicrobial property found in granules of phagocytising cells and in body fluids.

The lysozyme level in the blood serum was determined by a spectrophotometric measurement of clarification of a suspension culture of *Micrococcus lysodeicticus* in test tubes (Richter and Prochazkova, 1986).

## Data analysis

Statistics were calculated with MS-Excel 6.0® (means, SD) and GraphPad Prism software (inter-group differences). Statistical differences between groups were estimated by the unpaired nonparametric Mann-Whitney test. The differences with $P < 0.05$ and $P < 0.01$ were interpreted as significant and highly significant, respectively. Correlations between parameters were calculated according to the Spearman test.

Table 4. The level of phagocytosis of methacrylate particles, phagocytic index, level of chemiluminescence and lysozyme levels in dogs with chronic renal failure and healthy control (mean and standard deviation)

| Parameter | Control dogs | Group 1 | Group 2 | Group 3 | Subgroup 3 |
|---|---|---|---|---|---|
| Number | 15 | 10 | 15 | 20 | 10 |
| Clinical signs | none without azotaemia | none with azotaemia | mild | severe end-stage CRF | severe end-stage lymphopoenia |
| Phagocytosis (%) | 68.1 ± 29.4 | 64.4 ± 22.2 | 71.8 ± 23.9 | 68.8 ± 27.3 | 81.8 ± 20.2 |
| Phagocytic index | 21.8 ± 2.8 | 19.8 ± 3.3 | 17.4 ± 5.0 | 21.4 ± 1.6 | 20.3 ± 2.6 |
| Chemiluminescence – spontaneous (mV) | 2.9 ± 1.4 | 4.4 ± 4.1 | 4.5 ± 7.4 | 5.7 ± 10.6 | 2.8 ± 1.8 |
| Chemiluminescence – stimulated (mV) | 54.7 ± 22.4 | 97.9 ± 41.6** | 91.7 ± 66.6 | 80.7 ± 46.8 | 85.9 ± 51.6 |
| Chemiluminescence – index | 22.5 ± 10.9 | 30.5 ± 15.1 | 31.0 ± 21.2 | 31.6 ± 19.9 | 38.1 ± 17.8* |
| Lysozyme (g/l) | 4.7 ± 5.0 | 9.2 ± 5.2* | 9.7 ± 5.9* | 14.6 ± 6.6*** | 11.9 ± 6.7* |

*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ when compared with control dogs

## RESULTS

### Total and differential leukocyte counts

The total and differential leukocyte counts in each group of dogs with chronic renal failure and healthy control are summarized in Table 3. The main statistically significant findings were neutrophilia, lymphopoenia and a decrease in the number of eosinophils in groups of dogs with clinical signs (Groups 2 and 3).

### Phagocytosis of methacrylate particles, chemiluminescence test

The results of phagocytosis tests are summarized in Table 4. We did not find statistically significant differences between groups in the test of ingestion of methacrylate particles.

We found statistically significant difference in stimulated chemiluminescence between dogs with chronic renal failure without clinical signs and healthy control. Other groups of dogs with CRF had higher values of stimulated chemiluminescence as well; but the differences were not significant due to wide range of values. The values of both tests are shown in Table 3.

### Lysozyme level

The level of lysozyme was increasing with the extent of clinical signs (Figure 1, Table 4.) while this increase was statistically highly significant.

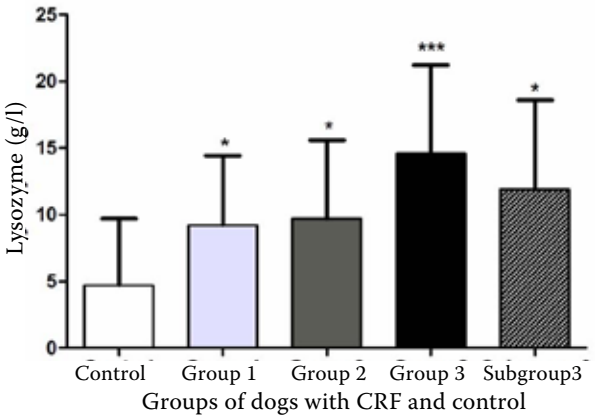


Figure 1. Lysozyme levels (g/l) in the control group and in each group of dogs with chronic renal failure

*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ when compared with control dogs

### Correlation between parameters

The levels of lysozyme are in significant positive correlation with the level of creatinine ($r = + 0.4904$, $P = 0.0006$, ***), urea ($r = + 0.3550$, $P = 0.0137$, *) and phosphorus ($r = + 0.5171$, $P = 0.0003$, ***). The number of red blood cells was in positive correlation with the percentage proportion of neutrophils ($r = +0.3104$, $P = 0.038$, *). There was a negative correlation between the number of lymphocytes and the level of phagocytosis ($r = –0.3629$, $P = 0.0143$, *).

## DISCUSSION

In this study we found that uraemia in dogs does not affect the neutrophil function in contrast with reports from human medicine. Statistically significant findings were neutrophilia, lymphopoenia, a decrease in the number of eosinophils, and a higher lysozyme level. Possible explanations of the differences between people and dogs are discussed.

Former reports on dogs discussed leukocytosis (DiBartola et al., 1989) and even leukopoenia (Nairn et al., 2005). We did not find any statistically significant change in the total leukocyte number.

We found an increased number of neutrophils in groups with clinical signs of chronic renal failure. Reports from human medicine discussing the number of neutrophils are controversial. They found a decreased number of neutrophils (Nairn et al., 2005), unchanged neutrophil counts (Alvarez-Lara et al., 2004) and an increased number of neutrophils in comparison with the healthy control (Fernandez-Fresnedo et al., 2000).

Our finding of lymphopoenia is consistent with previous reports both from human and veterinary medicine (Chew et al., 1983; Yoon et al., 2006). According to reports from human medicine, lymphopoenia may result from higher apoptosis of these cells, but the proper mechanisms are still unclear (Fernandez-Fresnedo et al., 2000; Jaber et al., 2001). In dogs with CRF and clinical symptoms we observed a statistically highly significant reduction of eosinophils in comparison with the healthy control. It does not correspond with previous reports from human medicine, where eosinophilia is common (Gabizon et al., 1981; Hallgren et al., 1984; Lee et al., 1995). Most patients with eosinophilia were haemodialysed, so haemodialysis per se was considered as a possible cause of the increased number of eosinophils (Bodner et al., 1982).

There is a lot of reports on neutrophil dysfunction in people with chronic renal failure, but the mechanisms responsible for reduced neutrophil functions are not well understood, although a number of partly interdependent factors have been proposed, including iron overload, anaemia, malnutrition and dialysis therapy per se (Lewis and VanEpps, 1987).

Iron is used in conjunction with recombinant human erythropoietin in the therapy of anaemia following chronic renal failure. Iron is required for several immune processes and in contrast it is necessary for bacterial growth. Iron overload may cause abnormalities of the neutrophil phagocytic function. The negative effect of iron on the neutrophil phagocytic function could be caused by a direct action of iron on the membrane or indirectly via enhanced production of oxygen metabolites (VanAsbeck et al., 1984; Himmelfarb and Hakim, 1994). We can exclude the influence of iron because none of the dogs in our study had received erythropoietin and iron therapy.

Anaemia affects the tissue oxygenation and may subsequently have negative effects on various biological functions. A highly significant correlation between the neutrophil function and hematocrit was observed and the neutrophil carbon dioxide production was improved with rHuEPO therapy (Vanholder and Ringoir, 1992). We found only a significant positive correlation between the number of red blood cells and the percentage proportion of neutrophils. But we did not find any statistically significant correlation between erythrocytes and neutrophil function.

The uremic condition is characterized by the progressive retention of numerous solutes; therefore phagocytic dysfunction by toxic suppression is likely to occur. The main potential toxins are polyamines (spermine and spermidine), glycated proteins, free immunoglobulin light chains and leptin. Spermine and spermidine are biogenic polyamines and they are well-known uremic toxins with a negative effect on erythropoiesis. They may interact with erythropoietin at a cellular level in the bone marrow (MacDougall, 2001). It was shown that spermine and spermidine suppressed the human neutrophil locomotion (Ferrante, 1985). Advanced glycation end products are elevated in uremic plasma and cause the increased neutrophil apoptosis (Cohen et al., 2001). In patients with the impaired kidney function there is an elevation of plasma levels of free immunoglobulin light chains (IgLCs). It was shown that IgLCs obtained from dialysed patients significantly inhibited chemotaxis and glucose uptake of neutrophils (Cohen et al., 1995). The measurement of the polyamine, advanced glycation end products and IgLCs levels was not the purpose of this study, but it is likely that their level increased with the worsening function of kidneys, i.e. with a higher level of urea, creatinine and phosphorus. However we did not find any correlation between the level of azotaemia and neutrophil counts and functions.

Leptin is another solute believed as uremic toxin and interfering with the function of neutrophils. Leptin is a nonglycosylated peptide hormone involved in the control of food intake. The molecule of leptin is similar to molecules of some cytokines (IL-6, IL-11, IL-12) and consistently with this view it plays a role in affecting the immune response. The serum from patients with CRF was found to inhibit the migration of normal neutrophils in response to N-formyl-methionyl-leucyl-phenylalanine (chemoattractant) with a strict correlation between serum leptin levels and serum ability to suppress the neutrophil locomotion (Ottonello et al., 2004). Leptin is produced by adipocytes and its level is in strict correlation with body mass. Patients with chronic renal failure often suffer from malnutrition and cachexia, so lower levels of leptin can be expected. But chronic renal failure in people is characterized by hyperleptinaemia due to the impaired excretion function of kidneys (Clark and Gao, 2002). Dogs and cats with chronic renal failure starve as well; cachexia and malnutrition are common findings. But the levels of leptin in dogs with CRF need further investigations because we did not find any statistically significant disturbance of neutrophils.

The difference between reports from human medicine and our findings may be caused by the fact that most of the former studies were performed with patients on dialysis therapy. It was proposed that haemodialysis per se may partly evoke disturbances in phagocytosis (Vanholder et al., 1993; Sardenberg et al., 2006). Nevertheless, there are some studies with non-haemodialysed uremic people, where the phagocytic function was impaired (Sharma et al., 2000).

An increased level of lysozyme in dogs with CRF is probably the main finding of this study; the level was increasing with clinical signs. We also found a strong positive correlation between the level of lysozyme and that of creatinine, phosphorus and

urea. This is in relation with Waldman et al. (1972). Lysozyme is an enzyme which destroys bacterial peptidoglycans and is found in high concentrations in neutrophil granules. It is a low molecular protein, so with decreased renal function there is retention of these molecules. The reports from human medicine are controversial: Kagan et al. (1989) found the elevated serum activity of lysozyme in patients with CRF undergoing continuous ambulatory peritoneal dialysis, but Sharma et al. (2000) did not observe any change in the lysozyme level in non-dialyzed people with chronic renal failure. There is a theory that the elevation in lysozyme levels is associated with degranulation of blood neutrophils and it might be a sign of the immune system activation (Trznadel et al., 1988). Despite these reports we tend to the opinion that the increased level of lysozyme is a result of decreased renal function according to the high positive correlation between levels of lysozyme, creatinine and urea.

Although we did not find any changes in phagocytic activity, we proved the negative influence of chronic renal failure on the activity of immune system in these groups of dogs with chronic renal failure. The negative effect was expressed by changes in lymphocyte counts and activity (Kralova et al., 2009, in press).

## CONCLUSION

Despite the reports from human medicine chronic renal failure in dogs did not affect phagocytosis. Changes in neutrophil counts and increasing lysozyme levels were the most significant results of this study. The changes in neutrophil counts were not associated with the changes in their function. The difference between dogs and humans may be caused by using dialysis therapy in uremic people, because dialysis per se may influence the immune status.

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