CLINICAL VALUE OF NITROBLUE TETRAZOLIUM TEST (NBT) IN THE DIAGNOSIS OF CANINE INFLAMMATORY PROCESSES

VALOR DO TESTE DE REDUÇÃO DO TETRAZÓLIO NITROAZUL (NBT) NO DIAGNÓSTICO DE PROCESSOS INFLAMATÓRIOS CANINOS

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SUMMARY

The purpose of the present study is to determine the clinical value of nitroblue tetrazolium reduction test (NBT) to diagnose inflammatory processes in dogs, and compare to white blood cell count (WBC) and concentration of plasma fibrinogen. To this end, we determined the percentage of NBT reducing neutrophils, concentration of plasma fibrinogen and WBC of 242 dogs, including both healthy dogs and animals with inflammation processes, with or without liver disease, azotemia or hypogammaglobulinemias. The NBT reduction test confirmed 30.43% of inflammatory diseases in dogs. The combined use of NBT reduction test, plasma fibrinogen and WBC was more efficient and identified 84.35% of inflammatory processes. Hypogammaglobulinemia and liver disease did not alter the ability of neutrophils to reduce NBT in dogs with inflammatory diseases, but results suggest that azotemia may affect the ability of neutrophils to reduce NBT. It can be concluded that NBT reduction test has low sensitivity for the diagnosis of inflammatory diseases in dogs, but it is useful in cases where the fibrinogen and WBC are not able to detect inflammation.

KEY-WORDS: Fibrinogen. Inflammation. White blood cell count (WBC). Leukocyte function. Dog.

RESUMO

Objetivou-se estimar o valor clínico do teste de redução do tetrazólio nitroazul (NBT) no diagnóstico dos processos inflamatórios de cães, comparando-o com o leucograma e a concentração plasmática de fibrinogênio. Para tal, determinou-se a porcentagem de neutrófilos redutores de NBT, a concentração plasmática de fibrinogênio e o leucograma de 242 cães, incluindo animais sadios e com quadro inflamatório, portadores ou não de hepatopatias, azotemia ou hipogamaglobulinemias. O uso do teste de redução do NBT permitiu confirmar 30,43% dos processos inflamatórios em cães. O uso associado do teste de redução do NBT com a determinação do fibrinogênio plasmático e leucograma possibilitou uma maior eficiência diagnóstica, identificando-se 84,35% dos processos inflamatórios. Não foi verificado um efeito significativo da hipogamaglobulinemia e da hepatopatia sobre a capacidade do neutrófilo reduzir o NBT em cães com doenças inflamatórias, porém os resultados obtidos sugerem que a azotemia possa afetar a capacidade do neutrófilo em reduzir o NBT. Conclui-se que o teste de redução de NBT é pouco sensível para o diagnóstico dos processos inflamatórios em cães, porém útil nos casos em que o fibrinogênio e o leucograma não são capazes de detectar a inflamação.

PALAVRAS-CHAVE: Fibrinogênio. Inflamação. Leucograma. Função leucocitária. Cão.

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INTRODUCTION

Several situations can trigger an inflammatory response, including infections, tissue damage and cancer (PEPYS E HIRSCHFIELD, 2003). Inflammatory response primary components are cytokines, acute phase proteins and leukocytes (LIBBY, 2007; KIM et al., 2011). The systemic response is followed by hyperthermia and leukocytosis (HARR, 2004), where the neutrophils and monocytes are the main cells released during acute inflammation processes (KAPLANSKI et al., 2003).

The most commonly used laboratory tests in Veterinary Medicine to evaluate inflammatory processes are white blood cell count and determination of plasma fibrinogen, but these tests have poor specificity to distinguish different causes of inflammation (VECINA et al., 2006).

Fibrinogen is an acute phase protein synthesized by the liver, whose plasma concentration rises under the stimulating action of both interleukins (IL-1 and -6) and tumor necrosis factor released by the inflammatory process, it is, therefore, a good predictor of response to acute inflammation (ANDREWS et al., 1994). During this phase, the plasma concentration increases for several days, reaching a peak between the fifth and seventh day (WEISS & WARDROP, 2010).

Neutrophil leukocytosis is observed during inflammatory processes, the neutrophil:lymphocyte ratio increases and shifts left within approximately 72 hours (FELDMAN, 2000). This leukocyte response varies according to inflammation cause, intensity and location, as well as species and animal age (SCHULTZE, 2000).

Neutrophils are cells that make up the first defense line of the organism (THRALL et al, 2007), when activated by inflammatory mediators they produce several reactive oxygen species, including superoxide and its derivatives (HUIMIN et al., 2000) that work as bactericidal, virucidal and fungicidal (BABIOR, 2004).

Several decades ago Park et al. (1968) developed the cytochemical nitroblue tetrazolium (NBT) reduction test, which enables to quantify neutrophil superoxide production. Subsequently, Gordon et al. (1973) observed that bacterial infections stimulate neutrophil oxidative metabolism and advocate the use of the NBT reduction test in humans as laboratory screening procedure in order to help the differential diagnosis of febrile disorders. At the same time Poli et al. (1973) used the NBT reduction test in dogs affected with various diseases, and concluded that this test can also be used successfully to distinguish bacterial diseases from others.

Although, the NBT reduction test has low cost, is easy to perform and therefore can be used as a routine lab test in veterinary, there are few studies to determine the clinical value of this test in dogs. Studies suggest that liver diseases (FAN & ZHANG, 1997), azotemia (TREVELIN et al., 2008; SOEIRO, 2010; BARBOSA et al., 2010) and dysproteinemias (POLI et al.,1973) may interfere with neutrophil oxidative metabolism and, consequently, with the NBT reduction test.

This study aimed to evaluate neutrophil superoxide production in dogs using the NBT reduction test, and therefore, to determine the clinical value of this test to diagnose inflammatory processes by comparing it with WBC and plasma fibrinogen, taking into account the effect of liver diseases, azotemia and hypogammaglobulinemias.

MATERIAL AND METHODS

All animals underwent a general clinical examination, as recommended by Feitosa (2008), and the following laboratory tests: NBT reduction test by neutrophil (stimulated and unstimulated proof), white blood cell count (WBC), plasma fibrinogen (F), serum urea, creatinine, alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma-glutamyl transferase (GGT), alkaline phosphatase (FA), total bilirubin, direct and indirect albumin, cholesterol, total proteins (PPT) and globulins.

According to the clinical examination and laboratory evaluation, we selected 242 adult dogs aged 7 years old average, not recently treated with any drug or vaccine. The following groups were formed:

Healthy group (GS): formed by 48 healthy dogs that did not present any changes in the parameters of both general clinical examination and laboratory test that indicated inflammation, azotemia, liver disease and hypogammaglobulinemia.

Inflammation group (GI): formed by 116 dogs that presented inflammation process and/or left shift and/or hyperfibrinogenemia associated to a PPT/F ratio lower than 10.

Group inflammation + liver disease (GIH): formed by 34 dogs with the same characteristics of GI associated with liver disease, characterized by the change of serum biochemical profile (ALT, AST, bilirubin total, direct and indirect GGT, AF, PPT, albumin and cholesterol)

Group inflammation + azotemia renal (GIA): formed by 34 dogs with the same characteristics of GI associated with increased levels of serum urea and/or creatinine (greater than 2.5 times the normal values), excluding post-renal azotemia.

Group inflammation hypogammaglobulinaemic + (GIHG): formed by 10 dogs with the same characteristics of GI group and associated with hypogammaglobulinemia.

Blood samples of 10 mL were drawn from each animal using disposable hypodermic needle 25 x 0.8 mm, from which an aliquot of 1 mL was placed in sterile plastic tube containing 10 U of heparin[2], in order to perform the NBT reduction test. Another 0.5-mL aliquot was placed in plastic tube containing 0.5 mg of EDTA-sodium to determine WBC and plasma fibrinogen. The remainder of the blood collected (8.5

[2] Liquemine® 5000 U/mL, Roche, São Paulo - SP.

mL) was placed in a glass bottle without anticoagulant at room temperature to obtain serum for the biochemical analysis.

Superoxide production in the neutrophils was determined by NBT reduction test, the cytochemical method described by Park et al. (1968), with minor modifications. Briefly, 50 µL of an aqueous buffered solution of NBT[3] (1g/L) was added to the 50 µL of heparinized blood in the eppendorf tubes. After resuspension, the sample was kept at 37°C for 10 minutes, followed by another 10 minutes at room temperature (22°C). In order to control false negative, the same procedure was followed in the stimulated sample where 2.5 µL of a commercial stimulant based on a bacterial extract[4] was added. The percentage of NBT reducing cells was established from the 100 neutrophil counts.

The total leukocyte count was performed using a semi-automatic blood cell count[5]. The differential leukocyte count was performed on blood smears stained with Panotic Fast[6] using an optical microscope. The plasma fibrinogen concentration was determined by the indirect precipitation method by heat (56 – 58ºC) and subsequent reading by refractometry[7], as recommended by Thrall et al. (2007).

All serum biochemical tests were carried out at 37ºC using commercial reagents[8] and the reading was conducted using a automated biochemical analyzer[9] previously set with commercial calibrators10 and controls11.

Fractionation of serum protein by electrophoresis was performed in horizontal vat by means of semi-microapplication (1,5 µL - 9 mm) on cellulose polyacetate tape (2,5 x 16 cm), using 0.04M sodium veronal buffer in pH 8.6, as described by Ciarlini et al. (1994). The identification and quantification of the different electrophoretic fractions were performed using an automatic densitometer[12].

The means and medians as measures of central tendency and the amplitudes and standard deviations as measures of dispersion were calculated for all variables of WBC, NBT reduction test and fibrinogen concentration for all five experimental groups. To assess the differences between groups, the Kruskal-Wallis and Dunn multiple comparison tests were used. We determined the frequencies (%) of laboratory changes associated with the inflammatory process of each species. The correlations between the different variables were estimated by the Spearman coefficients. All statistical analysis were performed by the software[13], at $p<0.05$.

[3] NBT vial, catalog n° 840-10, Sigma Diagnostic, St Louis, USA.
[4] STIMULANT, catalog n° 840-15. Sigma Diagnostic, St. Louis, USA.
[5] CELM CC 510, CELM, São Paulo, Brasil.
[6] InstantProv, Newprov, Pinhais-PR.
[7] Refractometer ATAGO® SPR-T2, ATAGO Co., LTD, Japan.
[8] BioSystems S.A, Barcelona-Espana.
[9] BTS-370 PLUS, BioSystems S.A., Barcelona-Espana.
[10] BioSystems S.A, Barcelona-Espana.
[11] BioSystems S.A, Barcelona-Espana.
[12] Densidômetro DS-35, CELM, São Paulo, Brasil.
[13] SAS/STA Software, Statistical Analysis System Institute, 1997, USA.

RESULTS AND DISCUSSION

The results for the healthy group (Table 1) are in agreement with the reference values considered by Schultze (2000) for the WBC, by Poli et al. (1973) for the NBT reduction test and by Thrall et al. (2007) for plasma fibrinogen, which ensured the clean bill of health of dogs in this group.

There was a great variation in the WBC, plasma fibrinogen and NBT reduction test in all groups. The overlap of these values between groups (Table 1) indicates that these variables have limited value in the differential diagnosis of the various inflammatory conditions investigated.

There was spontaneous reduction of NBT as high as 91 and 89% for dogs of Groups GI and GIH; however, no significant difference was found between groups (Table 1). Poli et al. (1973) also reported increased percentage of NBT reducing neutrophils in dogs with inflammatory processes, but these authors did not consider interfering factors as liver disease, azotemia and immunodeficiency that could contradict the results of this study.

On the other hand, the zero percent NBT reducing neutrophils (Table 1) could be associated with the presence of inhibitors of the oxidative metabolism and/or inflammatory reactions unable to stimulate enough the neutrophil oxidative metabolism. It is also possible that in some of the studied cases, the stress caused by the pain released corticosteroids, which are known to inhibit the NBT reduction test. In this regard, several studies conducted with other species have demonstrated the inhibitory effect of corticosteroids on neutrophil oxidative metabolism (ROTH & KAERBELE, 1981; HOEDEMAKER et al., 1992). In this study, the inflammation did not increase neutrophil oxidative metabolism in most cases. Less than 1/3 of the inflammation cases (Figure 1) presented increased NBT reduction rate. This result may be related in part to the presence of large numbers of animals suffering from immunosuppressive diseases such as visceral leishmaniasis (32.62%) and ehrlichiosis (5.17%).

The vast majority of dogs with visceral leishmaniasis (84.62%) presented normal or diminished NBT value. Only 15.38% of dogs with this infection showed increased superoxide production. These results can be explained by the great capacity of *Leishmania sp.* to evade neutrophil phagocytosis (LASKAY et al., 2003; GÓMEZ-OCHOA et al., 2010). It has been shown *in vitro* that *Leishmania* sp. evasion mechanism prevents the respiratory burst, thus preventing also the formation of reactive oxygen species such as superoxide (LAUFS et al., 2002). The mechanisms that make the infection by *Leishmania* sp. activate or inhibit neutrophil oxidative metabolism is under investigation. Recently, it was found that the increase or decrease of NBT reduction rate in dogs naturally infected with visceral leishmaniasis is related to the parasite load of *Leishmania* sp. (Ciarlini et al., 2010).

Hasegawa (2005) showed that dogs infected with *Ehrlichia* retain the ability to reduce NBT during the acute phase. In the present study the increase of

NBT reduction rate occurred only in 16.6% of the dogs with erhlichiosis, suggesting that lower superoxide production can occur during the chronic form of the disease. Ear infections, folliculitis and Panosteitis did not increase NBT reduction rate. Poli et al. (1973) also observed that neutrophil oxidative metabolism is not activated in all inflammation cases and NBT reduction rate varies with the different inflammation causes, being higher in bacterial processes.

The NBT value of Group GIH did not differ from other groups, which contradicts the inhibitory effect of liver diseases on the oxidative metabolism of human neutrophils observed by Fan and Zhang (1997). The values of NBT reduction in animals of Group GIA did not differ significantly; however, they were two times lower than Group GI (Table 1), suggesting that the azotemic and uremic compounds compromise the neutrophil oxidative metabolism of dogs. Recently, it was found that the plasma of healthy dogs when enriched with urea reduces the neutrophil oxidative metabolism (TREVELIN et al., 2008) and that plasma (SOEIRO, 2010) and serum (BARBOSA et al., 2010) components of dogs with chronic renal failure also inhibit neutrophil superoxide production.

According to Cerone et al. (1997) the decrease of immunoglobulin affects the interaction between neutrophils and Fc receptors, and thus compromises the respiratory burst responsible for the increased superoxide production. The dogs of Group GIHG had the greatest NBT reduction rate, contrary to the reports by Hansen et al. (1995) and Leino & Paape (1996), who reported that hypogammaglobulinemia inhibits neutrophil oxidative metabolism. These results suggest that the activation of neutrophil oxidative metabolism in the studied inflammatory processes happened by other ways that are independent of the immunoglobulin-receptor interaction.

It was observed that 71.42% of the dogs with increased NBT reduction rate presented also inflammatory WBC (left shift); however, hyperfibrinogenemia with PPT/F lower than 10 was observed in only 7.14%. This suggests that in most of the inflammatory processes studied, the mechanism that induces hyperfibrinogenemia precedes the activation of neutrophil oxidative metabolism and leukocyte changes.

Hyperfibrinogenemia occurred in only 13.91% of the animals with inflammation (Figure 1), thus indicating that this parameter was less sensitive for the detection of inflammatory processes when compared to the WBC and NBT reduction test. According to Schalm et al. (1970) increased fibrinogen is not frequently observed in dogs with inflammation, probably because this protein is rapidly consumed during the initial stage of inflammatory processes.

In this study it was observed that 68.75% of the dogs with high concentrations of plasma fibrinogen (PPT/F < 10) had normal white blood cell count (WBC), similar to what has been reported by Sutton & Johnstone (1977) and Vecina et al. (2006). These results also support the concept that plasma fibrinogen evaluation is a valuable tool for clinical and laboratory diagnosis of the initial phase of inflammation in dogs, even when this condition is not identified by the WBC.

The left shift, the main indicator of inflammation, detected 61.73% of the cases compared to the NBT reduction test that detected 30.43% of the cases (Figure 1). The combined use of WBC and NBT reduction test detected 77.39% of the inflammatory processes, and this detection rate increased to 84.35% of the cases (Figure 1) when the tests were combined with fibrinogen assessment. However, even when combining NBT reduction test, WBC and fibrinogen, 15.65% of the dogs with inflammatory processes were not identified. These results emphasize how important it is for the veterinarian to conduct a careful overall clinical assessment of the patient, since some inflammatory processes are not accused by the routine laboratory tests.

CONCLUSION

The cytochemical NBT reduction test by neutrophils was not very effective to diagnose the inflammatory processes of dogs; however, it was useful in cases where the inflammation was not detected by increasing plasma fibrinogen and white blood cell count (WBC). Separately, hypogammaglobulinemia and liver diseases did not affect significantly the ability of the neutrophils to reduce NBT in dogs with inflammatory diseases, but there are indications that azotemia may affect the ability of neutrophils of reduce NBT.

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Table 1 - Statistical analysis results of NBT reduction test, plasma fibrinogen concentration and absolute leukocyte counts of healthy dogs (GS), and dogs with inflammation (GI), inflammation and liver disease (GIH), inflammation and azotemia (GIA), inflammation and hypogammaglobulinemia (GIHG).

		Mean	Standard deviation	Median	Amplitude
NBT(%)	GS	7.96	4.72	8 a	0 – 15
	GI	13.79	19.22	7 a	0 – 91
	GIH	12.82	20.72	8 a	2 – 89
	GIA	10.25	13.14	4.5 a	1 – 54
	GIHG	25.66	28.74	16 a	3 – 58
Fibrinogen (g/dl)	GS	0.21	0.13	0.2 a	0.1 – 0.5
	GI	0.42	0.26	0.4 b	0.1 – 1.4
	GIH	0.37	0.29	0.2 ab	0.1 – 1.1
	GIA	0.42	0.21	0.4 ab	0.1 – 0.9
	GIHG	0.5	0.34	0.7 ab	0.1 – 0.9
Leukocytes ($x10^3/\mu l$)	GS	11.12	3.03	10.50 a	6.6 – 17
	GI	12.20	6.6	11.45 a	1.7 – 37.8
	GIH	16.94	12.11	12.40 a	5 – 49.7
	GIA	12.77	12.77	12.05 a	2 – 29.1
	GIHG	19.20	19.2	11.70 a	7.6 – 38.3
Band neutrophil ($x10^3/\mu l$)	GS	0.22	0.46	0 a	0 – 0.16
	GI	0.55	0.87	0.22 b	0 – 4.75
	GIH	2.18	4.69	0.93 b	0 – 19.87
	GIA	1.19	1.66	0.3 ab	0.73 – 6.51
	GIHG	0.51	0.84	0 ab	0 – 1.53
Segmented ($x10^3/\mu l$)	GS	6.82	2.02	6.54 a	3.29 – 10.53
	GI	7.87	5.78	6.49 a	0.37 – 3.70
	GIH	11.10	7.18	7.99 a	2.90 – 24.75
	GIA	9.12	6.57	7.95 a	0.36 – 2.35
	GIHG	14.16	15.26	5.38 a	5.32 – 31.78
Lymphocytes ($x10^3/\mu l$)	GS	2.70	0.84	2.67 a	1.07 – 4.31
	GI	1.97	1.56	1.57 b	0.10 - 7.70
	GIH	1.61	1.43	1.18 b	0.05 – 4.97
	GIA	1.18	0.92	0.88 b	0.12 – 2.90
	GIHG	1.37	0.36	1.36 ab	0.98 – 1.75
Monocytes ($x10^3/\mu l$)	GS	0.56	0.37	0.50 a	0 – 1.53
	GI	0.97	0.83	0.76 ab	0 – 5.37
	GIH	1.63	2.39	0.99 b	0 – 10.5
	GIA	1.01	0.66	0.97 ab	0.06 – 2.76
	GIHG	1.59	1.57	1.21 ab	0.11 – 3.83
Eosinophils ($x10^3/\mu l$)	GS	0.92	0.66	0.88 a	0.06 – 2.85
	GI	0.65	0.80	0.4 ab	0 – 4.23
	GIH	0.26	0.34	0.19 b	0 – 1.32
	GIA	0.24	0.29	0.23 b	0 – 1.10
	GIHG	1.08	2.16	0 ab	0 – 4.32
N : L ratio	GS	2.91	1.907	2.2 a	1.3 – 9
	GI	8.55	15.41	4.0 b	0.3 – 98
	GIH	18.24	24.42	6.6 b	1.5 – 87
	GIA	16.76	24.84	7.45 ab	0.7 – 81
	GIHG	12.06	13.58	5.5 ab	3.1 – 27.7

* Different letters in the same column mean significant differences ($p<0.05$)

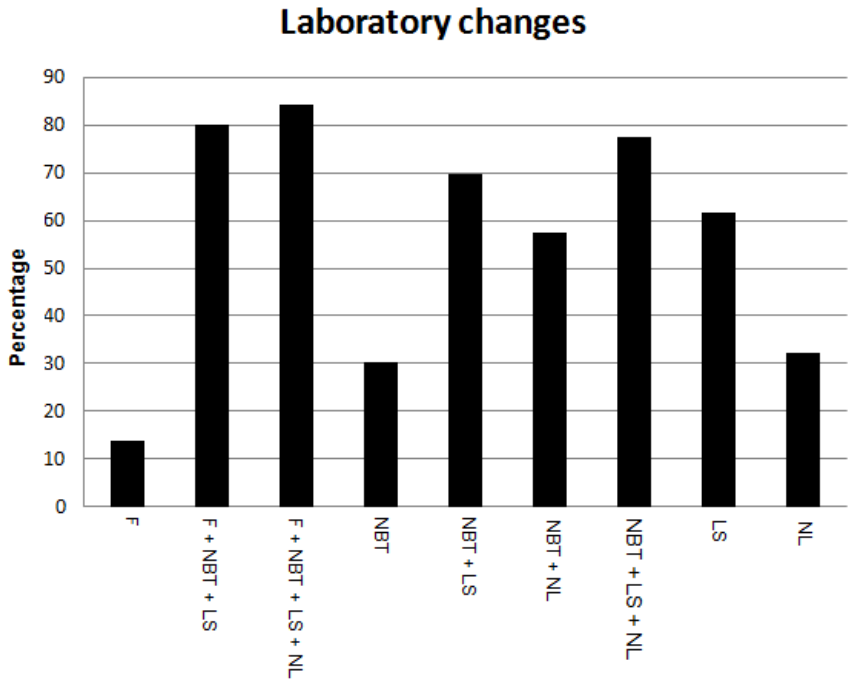

Figure 1 - Percentage of inflammatory processes of dogs detected by different laboratory tests, alone or combined: hyperfibrinogenemia (F), NBT reduction test (NBT), left shift (LS) and increase of the neutrophil : lymphocyte (NL) ratio.

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