



Acute Phase Proteins in Canine Lymphoma During Antineoplastic Chemotherapy

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Abstract

The lymphoma is the main hematopoietic tumor in dogs and it is characterized by the proliferation of cells from lymphoid tissue, histiocytes and its precursors. Animals with lymphoma often show changes in biochemical and hematological parameters such as non-regenerative normochromic normocytic anemia, hemolytic anemia, hypocalcaemia and monoclonal gammopathy. The development of tumor can cause alterations in serum concentrations of acute phase proteins (APPs), consequent of hepatocytes stimulus by cytokines of inflammatory action. This study aimed to quantify and qualify APPs in dogs with lymphoma, at diagnosis time and during the time of chemotherapy sessions. After syneresis, centrifugation and fractioning the serum samples of 10 healthy and 10 dogs with lymphomas, the proteins fractions were separated by polyacrilamide gel electrophoresis (SDS-PAGE) and its concentrations were determined by computer densitometry. Between 18 and 30 proteins were separated by electrophoresis, with molecular weights ranging from 18 to 245 kDa (kilodaltons). The alpha-1-glicoprotein acid (AGP) and transferrin serum concentration showed significantly higher in dogs with lymphoma, when compared with healthy dogs at diagnosis. The alpha-1-antitripsin (AAT) serum concentrations showed significantly higher in healthy dogs, when compared with dogs with lymphoma at diagnosis. The dogs with lymphoma the albumin did not appear as negative APP. On the other hand, transferrin appeared as positive AAP at diagnosis time and during the chemotherapy sessions. Healthy dogs had AAT serum concentrations significantly higher when compared to dogs with lymphoma at diagnosis. So, in this trial, it is suggested that this protein has been shown as a negative APP in the dogs with lymphoma. These dogs presented significantly higher AGP serum concentrations, in relation to healthy dogs at diagnosis, evidencing this protein APP positive behavior in neoplasm.

Key words: dog, cancer, protein, electrophoresis, chemotherapy

Introduction

Lymphoma is one of the most commonly diagnosed neoplasm in dogs and accounts for more than 80% of hematopoietic neoplasia in this specie (9). Lymphoma is characterized by malignant lymphoid cells proliferation, primarily affecting the lymph node or solid visceral organs such as liver and spleen. Its incidence is considerable in all domestic animals species, mainly dogs and cats (17).

Animal with lymphoma often have changes in hematological and biochemical indexes, such as normocytic normochromic non-regenerative anemia, hemolytic anemia, hypercalcaemia, and monoclonal gammopathy, often considered as paraneoplastic syndromes (10).

The antineoplastic chemotherapy is the main therapeutic approach for the dogs with lymphoma, since this is the tumor that better answer to this kind of treatment¹⁰. However, antineoplastic drugs can cause

several toxic effects, especially to the cellular systems that are constantly dividing, such as the hematopoietic tissue. Because of this, animals under chemotherapy are monitored periodically along the treatment (2).

The sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) represents one of the most reliable methods to identify fluid body proteins (15). The neoplasm development may cause changes in acute phase proteins (APPs) serum concentrations, due to the hepatocytes biosynthetic stimulation by the action of inflammatory cytokines (11). The acute phase is characterized as an initial response to different forms of injury, bacterial invasion, localized or systemic inflammation (12). Those proteins are glycoprotein regulated by cytokines such as tumor necrosis alpha factor, interleukin-1 and interleukin-6 (20). When an APP presents its concentration increased in the serum or plasma is considered a positive APP (alpha-1-antitrypsin, alpha-1-acid glycoprotein, ceruloplasmin and haptoglobin), and when its concentration decreased it is considered a negative APP (prealbumin, albumin and transferrin) (11).

The different protein fractions separation is achieved due to the protein molecule electric charge, protein size, alkaline pH of the medium, and electrophoretic running buffer. The electrophoresis is characterized by migration of protein in an electromagnetic field under different speeds (24).

Dogs with lymphoma present important APPs alterations (11). Therefore, we conceived this assay aiming mainly evaluate these changes right from the lymphoma diagnosis to the end of the chemotherapy protocol, using the SDS-PAGE technique.

Material and Methods

Institutions Involved

This assay was carried out at FCAV, UNESP, - Veterinarian Hospital, Veterinarian Oncology Sector, Clinical Pathology Laboratory, as well by the Veterinarian Clinic and Surgery Department - Research Laboratory.

Experimental Groups

A total of 20 dogs, male or female, distributed in two different groups. In the control group (CG) there were 10 healthy dogs and in the lymphoma group (LG) there were 10 dogs with lymphoma diagnosis established on clinical exam, tissue or organ cytology and/or histopathology. (Protocol of Animal Ethics Committee – CEUA FCAV Unesp - n° 018137- 08)

Antineoplastic Chemotherapy

It was established based on the Madison-Wisconsin Protocol (25), using only the ten weeks induction phase, consisted of the following drugs: vincristine sulfate: 0.75mg m² intra-venous dose, at 1st, 3rd, 6th, and 8th weeks, L-asparaginase: 400 IU kg⁻¹ intra-muscular dose in a single dose at 1st week,

cyclophosphamide: 250mg m² oral dose at 2nd and 7th weeks; doxorubicin: 30 mg m² intra-venous dose at 4th and 9th weeks; prednisone: of 2 mg kg⁻¹ day, oral dose at 1st week, for seven days, and then 1.5 mg kg⁻¹ day at 2nd week, by seven days, and then 1 mg kg⁻¹ day at 3rd week, for seven days, and finally, 0.5 mg kg⁻¹ day at 4th week, for seven days. Dogs with lymphoma were evaluated during the chemotherapy per nine weeks. At the 5th and 10th weeks were the chemotherapy interval.

Blood Collect

Blood samples were obtained by venipuncture or cephalic vein with 25x7 syringes of five mL and hypodermic needles after local antisepsis, and stored in test tubes (10x70) without anticoagulant.

Samples Preparation

The blood was centrifuged for five minutes at 1257.6g (2500 rpm). Immediately thereafter, the serum was packaged and frozen stored at -18°C up to the electrophoretic analysis moment. Healthy dogs' samples were obtained only once, and the Lymphomatoid dog samples were obtained at the diagnosis moment and weekly, just before each chemotherapy session during nine weeks, totalizing eight collecting.

Laboratorial Analyses

Total Serum Proteins

The total serum proteins were determined through the Biuret's method with a set of commercial reagents (Labtest®, LabQuest®) and reading in a semi-automatic spectrophotometric.

Electrophoretic separation

The SDS-PAGE was performed as described by LAEMMLI (1970) (16) modified, using the vertical electrophoresis system (PROTEAN II XI-VERTICALELECTROPHORESIS CELLS® - BIO-RAD). The gel polymerization was possible by adding 15.0 mL of tetramethylethylenediamine (TEMED) and 0.3 mL of persulphate of ammonia 10%. The plate was placed in an appropriate support, in contact with the buffer solution under pH 8.5 and subjected to a 20 mA electric current. After the separation, the gel was stained for two hours in a Coomassie Brilliant Blue 0.2% solution in the horizontal shaker to achieve an uniform coloration and then the excess stain was removed using a bleach solution. The molecular weights and concentrations of protein fractions were determined by computer densitometry (Shimadzu CS-9301) by scanning the samples. For the protein identification markers were used (SIGMA MARKER™, wide range, from 6.5 to 200 kDa) of 200, 116, 97, 66, 55, 45, 36, 29, and 20 kilodaltons (kDa) molecular weights, and also albumin, alpha-1-antitrypsin, haptoglobin, ceruloplasmin, transferrin, and

IgG purified proteins. To the densitometry evaluation of protein bands were done reference curves from the standard marker reading.

Statistics

It was used the Variance Analysis by F test to verify if the APPs differ between group of dogs healthy and with lymphoma at diagnosis and between lymphoma dogs during the chemotherapy sessions. The Tukey test at 5% probability was used for comparison of means between lymphoma bearing and healthy dogs and between dogs with lymphoma during the different chemotherapy sessions. It was used the AGROESTAT (version 1.0, 2008) (1) statistical program to do these analysis.

Results

The SDS-PAGE technique allowed fractionate proteins, whose molecular weights ranged from 18 to 245 kDa (Figure 1). For the healthy and lymphomatoid animals studied in this trial, eight proteins were important in protein electrophoresis, because showed heavy marked in gel of electrophoresis. Six proteins were identified by name, being two proteins presented as positive APP (alpha-1-acid glycoprotein and transferrin), one as negative APP (alpha-1-antitrypsin), and albumin, ceruloplasmin and haptoglobulin presented with normal value. Two proteins did not identified by name, also they were presented as proteins 23 kDa and 33 kDa.

The albumin, ceruloplasmin, and haptoglobin serum concentrations (g dL⁻¹) mean between lymphomatoid and healthy dogs at diagnosis, and between dogs with lymphoma before each chemotherapy session, did not present significant differences (p> 0.05) (Tables 1 and 2). The alpha-1-antitrypsin, alpha-1-acid glycoprotein, and transferrin serum concentrations (g dL⁻¹) means between lymphomatoid and healthy dogs at diagnosis, were significantly different (p<0.05) (Table 1 and Figure 2), and between dogs with lymphoma before each chemotherapy session, did not present significant differences (p> 0.05) (Table 2).

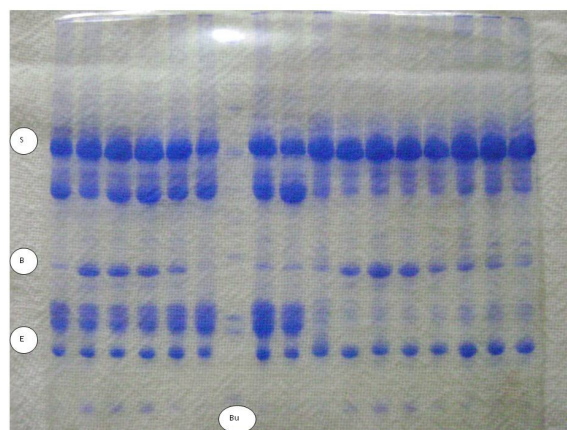


Figure 1. Electrophoresis of serum proteins in SDS PAGE showing separating of acute phase proteins in dogs. There are 17 samples of blood serum, being the buffer sample (Bu). The proteins with high molecular weigh locate in start (S) of the electrophoresis, being between it's as protein ceruloplasmin (125 kDa), transferrin (85 kDa), albumin (65 kDa), alpha-1 antitrypsin (60 kDa). In between (B) of the gel are the proteins haptoglobin (39 kDa) and alpha-1-acid glycoprotein (37 kDa). For the end (E) of the gel are the proteins n° 9 (33 kDa), and protein n° 11 (23 kDa).

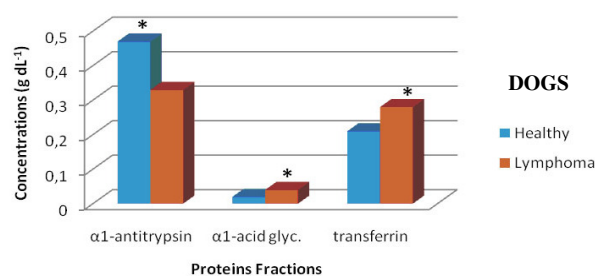


Figure 2. Alpha-1-antitrypsin, alpha-1-acid glycoprotein, and transferrin proteins fractions serum concentrations (g dL⁻¹) graphical representation verified in the SDS-PAGE, which showed significant changes (*) in healthy and dogs with lymphoma at the diagnosis time.

Table 1. Acute phase proteins serum concentrations (g dL⁻¹) means and standard deviations verified at SDS-PAGE in healthy and dogs with lymphoma at diagnosis.

Protein	Molecular Weigh (kDa)	Healthy Dogs (n=10)	Dogs with lymphoma (n=10)
Albumin	65	3.62±0.26	3.51±0.64
Transferrin	85	0.21±0.02 ^a	0.28±0.11 ^b
α1-antitrypsin	60	0.47±0.13 ^a	0.33±0.16 ^b
α1-acid glycoprotein	37	0.02±0.01 ^a	0.04±0.03 ^b
Ceruloplasmin	125	20,14±12,12 (x10 ⁻³)	15,34± 9,33 (x10 ⁻³)
Haptoglobin	39	13,20±4,77 (x10 ⁻³)	22,69±15,36 (x10 ⁻³)

Means followed by different letters in the same row differ (p<0.05) by Tukey test.

Table 2. Acute phase proteins serum concentrations (g dL⁻¹) overall averages and standard deviations, verified at SDS-PAGE, in dogs with lymphoma before each weekly chemotherapy week session.

Weeks	Albumin	Transferrin	α 1-antitrypsin	α 1-acid glycoprotein	Ceruloplasmin (x10 ⁻³)	Haptoglobin (x10 ⁻³)
1 st	3.51±0.64	0.28±0.11	0.33±0.16	0.04±0.03	15,34±9,33	22,69±15,36
2 nd	3.84±0.62	0.25±0.10	0.34±0.11	0.02±0.02	17,25±12,21	31,95±16,62
3 rd	4.16±0.76	0.25±0.80	0.45±0.08	0.02±0.01	13,53±11,68	38,62±21,43
4 th	3.80±0.92	0.23±0.10	0.38±0.06	0.02±0.01	16,28±12,71	40,47±21,44
6 th	3.99±0.43	0.25±0.09	0.42±0.12	0.04±0.03	7,11±7,79	34,03±18,87
7 th	3.76±0.67	0.30±0.12	0.41±0.21	0.04±0.02	15,45±19,29	33,04±28,11
8 th	3.70±0.77	0.24±0.12	0.43±0.18	0.04±0.03	18,51±26,56	32,40±19,32
9 th	3.42±0.83	0.25±0.11	0.39±0.14	0.04±0.03	25,99±47,97	29,56±15,23

The 5th and 10th weeks are the chemotherapy intervals.

Discussion

By the biological significance and multiple functions performed in the organic system, the total protein and its fractions (albumin, alpha, beta and gamaglobulin) serum levels evaluation obtained by electrophoresis, is an important help to the clinic diagnosis (15). The neoplasm development may cause changes in the APPs serum concentrations, due to the hepatocytes biosynthetic stimulation by inflammatory cytokines action (11).

The reference values to serum albumin concentration in dogs are between 2.5 to 4 g dL⁻¹ (4). The results obtained to serum albumin rates in this study showed that both healthy and dogs with lymphoma the values were within the reference range to the specie. Albumin is considered a negative AAP in sick dogs and even without significant difference (p > 0.05) in the comparison between healthy and dogs with lymphoma and between treated and untreated with chemotherapy protocol, it was lower in dogs with lymphoma, corroborating by Eckersall (11).

In dogs with distemper, it was evidenced an increase in the albumin rate, a decrease in alpha and betaglobulins fractions, and a significant increase at immunoglobulin fraction (27). In this study, the dogs with lymphoma did not presented an elevation at albumin serum concentration, but presented an increased at AGP, transferrin and IgG serum concentrations. The serum albumin concentration increased slightly (from 7 to 13%) in dogs after the glucocorticosteroids administration (21). Specifically in this study, it was also observed that the albumin serum concentrations increased slightly after the 1st, 2nd, and 4th glucocorticosteroids (prednisone) administration in dogs with lymphoma. The APPs have the ability to link to the pharmacological agents and the reports show that the drugs disposition, distribution and kinetics in the animal organism are significantly altered during the acute phase in the inflammatory process. Thus, the drugs effectiveness may be altered (14).

According Nakage (23), healthy dogs presented a significant decrease in the albumin serum concentrations, after three doxorubicin administrations. In this trial, dogs with lymphoma did not show significant difference in serum albumin concentrations, after two administrations of doxorubicin.

Hepatotoxicity resulting from antineoplastic agents, in a general way, is diagnosed through the enzymes such as alanine aminotransferase (ALT), alkaline phosphatase (ALP), bilirubin, and hypoalbuminemia serum activities elevation (25). However, in this study, it was not verified decrease in the albumin serum concentration in dogs with lymphoma after the chemotherapeutic protocol. L-asparaginase inhibits the lymphocytes T and B function, further on lead the dogs to a hepatotoxicity with consequent increase in the serum activities of the ALT and aspartate aminotransferase enzymes, bilirubin and serum albumin serum activities (25). In this test there was an increase in the serum albumin after L-asparaginase administration, but without significant changes among the dogs with lymphoma both treated or untreated.

In sick dogs the transferrin is considered a negative APP (11). However, in this test it behaved as a positive AAP, presenting significant difference between healthy and dogs with lymphoma at the diagnosis, with significant higher (p<0.05) concentrations in dogs with lymphoma, disagreeing directly to the findings of Eckersall (11), that reported decrease of this protein in neoplasm. The transferrin serum concentration was elevated in broilers with inflammatory and infectious diseases, suggesting that in broilers the transferrin is also a positive AAP (29). Transferrin binds to iron to be carried through the tissues, and high serum concentrations may be related to iron deficiency²⁷. Besides, the transferrin concentrations may increase the liver disease and nephrotic syndrome and decrease iron storage (10).

Alpha-1-antitrypsin (AAT) is considered positive AAP in sick dogs (11), but its serum

concentrations were significantly lower ($p < 0.05$) in dogs with lymphoma, behaving as negative APP, contrary to Thomas (28), who observed high alphasglobulin peaks in dogs with several neoplasm. The proteins that migrate to the alphasglobulins region are the APPs that increase immediately after an inflammation or infection (6), fact that disagrees with this current study, regarding to the AAT. Moreover, the AAT may have its serum concentrations decreased the liver disease and chronic pulmonary disease (11). Lower values of AAT are associated with reduced intensity of inflammation (22), which contradicts these test findings, since the dogs with lymphoma had lower AAT serum concentrations, in relation to healthy dogs.

Alpha-1-acid glycoprotein (AGP) is considered positive APP in sick dogs, being significantly higher ($p < 0.05$) in dogs with lymphoma at the diagnosis, corroborating by Eckersall findings (11), who reported this protein elevation in neoplasm. The AGP concentrations determination may be useful in estimating the tumor load, comparing dogs with lymphoma treated or untreated with doxorubicin (18). The AGP serum determination was useful in monitoring dogs with lymphoma treated with doxorubicin, because the AGP concentration decreased significantly in animals with complete remission and rose again three weeks before the lymphoma relapse (6). Dogs with mammary tumors and other neoplasm did not present high AGP serum concentrations, when compared with healthy dogs, although is believed that the AGP molecule may increase the sialic acid concentration in neoplastic states. Elevations in the serum levels of this protein have been described in dogs with lymphoma, carcinomas (except breast carcinoma) and sarcomas (6).

The AGP serum concentration is able to estimate the tumor load and its changes allowed predict the lymphoma relapse weeks before the clinical manifestation of dogs with lymphoma treated with doxorubicin (8). But in this study, there were not significant differences ($p > 0.05$) in the AGP serum concentrations between dogs with lymphoma treated with doxorubicin, disagreeing with the Hahn's findings (13).

The ceruloplasmin is considered a positive AAP in sick dogs (11), but in this study, there was not significant difference ($p > 0.05$) between healthy and dogs with lymphoma at diagnosis, presenting a slight reduced in dogs with lymphoma, a fact that disagree with Calazans (5) who reported significant differences when were compared dogs with lymphoma in the seventh day of chemotherapy. Studies with lymphoma dogs revealed that the ceruloplasmin and haptoglobin proteins had high serum concentrations when compared to healthy animals (8), disagreeing with the findings of this study.

The haptoglobin acute phase profile differs between species. In canine, feline, equine and swine species, the reference values are between 0.1 and 0.2 g dL⁻¹, however, in inflammatory and infectious diseases it can increase to 0.5 g dL⁻¹ or even more (11). In this test, the mean haptoglobin serum concentrations to

healthy and dogs with lymphoma at diagnosis were 0.013 and 0.022 g dL⁻¹, respectively, both below the reference values of specie. The serum concentrations mean (g dL⁻¹) of haptoglobin in dogs with lymphoma during the weekly sessions of chemotherapy did not present significant differences ($p > 0.05$), corroborating with Calazans' reports (5), who also did not observe such difference.

Haptoglobin is considered a positive AAP in sick dogs (11), increasing in the acute phase of the inflammation. In this study, even without significant difference ($p > 0.05$) it was observed higher haptoglobin serum concentrations in dogs with lymphoma, corroborating to the Mischke & Eckersall data (20). Although haptoglobin in dogs has been recognized as moderate APPs belonged protein (7), some studies have shown changes in this protein concentration, with a possible prognostic and diagnostic indication in the inflammations (18).

The canine haptoglobin is particularly sensitive to glucocorticosteroids, and presents an increase in its serum concentration after administrate prednisone of an oral dose of 2 mg kg⁻¹, for three consecutive days (19). The prednisolone glucocorticosteroid is included in the list of drugs used at chemotherapy protocol in the present experiment, being administrated in first month of chemotherapy sessions. It was verified that after the two first sessions there was an increase in the haptoglobin serum concentration, but without exceed the normal ranges, confirming the findings of Martinez-Subiela (19), who reported elevations in the haptoglobin levels after the glucocorticosteroids administration.

Conclusions

In dogs with lymphoma the albumin did not appear as negative or positive APP and transferrin appeared as positive AAP at diagnosis time and during the chemotherapy sessions. The transferrin serum concentrations were significantly elevated in lymphoma dogs, suggesting that in lymphoma this protein can be high or the tumor induce iron deficiency.

Dogs with lymphoma had AAT serum concentrations significantly lower at diagnosis when compared to healthy dogs. This suggests that this protein is as a negative APP in the dogs with lymphoma. These dogs presented significantly higher AGP serum concentrations, in relation to healthy dogs at diagnosis, evidencing this protein APP positive behavior in lymphoma. Haptoglobin and ceruloplasmin showed no significant difference..

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