JORNAL INTERDISCIPLINAR DE BIOCIÊNCIAS

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Cytological analysis of bronchoalveolar lavage fluid of dogs with Visceral Leishmaniasis and infused with bone marrow mononuclear cells

Análise citológica do fluido do lavado broncoalveolar de cães com Leishmaniose Visceral e infundidos com células mononucleares de medula óssea

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A B S T R A C T

The respiratory system has a direct contact with external environment, but this anatomical feature can be the entry route to many agents of injury. Lung disorders are not rare in clinical practice and analysis of bronchoalveolar lavage fluid (BALF) becomes an useful tool to assess possible changes in lung cell population against local injury, cellular embolism and after infusion of bone marrow mononuclear cells (BMMC) intravenously (IV). We evaluated qualitatively and quantitatively the cellular components of BALF from dogs naturally infected by *Leishmania infantum chagasi* and compared with infected animals and infused with BMMC. Thirteen animals were divided into negative control group (N = 6 dogs) and naturally infected (N = 7 dogs), and four dogs in this group were infused IV with BMMC homologously. The collect of BAL was performed with endotracheal tube; BMMC were obtained upon puncture of the iliac crest, separated by density gradient and adjusted a final volume containing 1×10^8 cells to IV infusion in each dog. The data obtained showed a significant difference in total count of cells between control and naturally infected group, showing a decrease in the pulmonary cell population in animals with leishmaniasis. The treated group showed an increase in the total of cells presents in the BALF from each dog, but the overall average there was no significant difference. In the same group there was a decrease in polymorphonuclear cells count and an increase of mononuclear cells ($p \leq 0.05$). The cellular analysis of BALF showed that pulmonary immune response is significantly depressed in canine visceral leishmaniasis and mononuclear cells population rises after BMMC infusion.

K E Y W O R D S

Dogs; Visceral leishmaniasis; Stem cells; Cytology

R E S U M O

O sistema respiratório apresenta contato direto com o ambiente externo, e esta característica anatômica torna-se uma porta de entrada para diversos agentes agressores. Alterações pulmonares não são raras na rotina clínica e a análise do fluído do lavado broncoalveolar (FLBA) torna-se uma ferramenta útil para avaliar possíveis alterações na população celular pulmonar frente a situações de injúria local, embolia celular e após infusão IV de células mononucleares da medula óssea (CMMO). Objetivou-se avaliar qualitativamente e quantitativamente os componentes celulares no FLBA de cães naturalmente infectados pela *Leishmania infantum chagasi* e comparar com animais infectados e infundidos com CMMO. Foram utilizados treze animais, separados em grupos controle negativo (N=6 cães) e infectados naturalmente (N=7 cães), sendo que quatro cães deste grupo foram infundidos IV com CMMO homologamente. A coleta do FLBA foi realizada com sonda orotraqueal; as CMMO foram obtidas diretamente por punção da crista ilíaca, separadas por gradiente de densidade e ajustada a concentração de $1x10^8$ células para infusão IV em cada cão. Os dados obtidos do FLBA revelaram uma diferença significativa no número total de células entre os grupos controle e naturalmente infectado, mostrando um decréscimo nos animais com leishmaniose. O grupo tratado revelou um aumento no total de células presentes no FLBA de cada cão, mas na média geral não houve diferença significativa. Neste mesmo grupo houve um decréscimo na contagem de células polimorfonucleares e um incremento de células mononucleares ($p \leq 0,05$). As análises celulares do FLBA revelaram que a resposta imune pulmonar na leishmaniose visceral canina está significativamente deprimida e a população de células mononucleares eleva-se após a infusão de CMMO.

P A L A V R A S - C H A V E

Cães; Leishmaniose visceral; Células-tronco; Citologia

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INTRODUCTION

The respiratory system has direct contact with the environment and this anatomical feature is a major factor to expose the upper and lower respiratory tract to external aggressions (BASSO, 2008). One of the main characteristics observed in lung disease is inflammation, which can cause the acute inflammatory process evolve into a critical state: the distress syndrome, bronchiolitis obliterans, chronic obstructive disease and cystic fibrosis (ADAGE et al., 2015).

In small animals clinic routine it is observed that 4% of cases are associated with lung disorders (FERIAN et al., 2006). The complementary test more affordable, non-invasive and quite used to these conditions it is the chest radiograph. However, its resources are not specific to establish a diagnosis (NORRIS et al., 2002).

In general, respiratory diseases are difficult to detect, thus, additional tests are necessary: blood count, biochemical, blood gas analysis, serology, radiographs, fluid analysis of the airways and lung biopsy (SILVERSTEIN and DROBATZ, 2005; FERIAN et al., 2006).

An examination to be considered to assess the early diagnosis of pulmonary disease is the bronchoalveolar lavage method (BAL), a tool that assesses the cellular components of the lower airways from fluid collected within a bronchial segment and alveolar (FORGARTY and BUCKLEY, 1991; NELSON and COUTO, 1994; CLARK et al., 1995).

Obtaining fluid allows a cytological and microbiological exploration (BASSO, 2008; RIBAS, 2010). The main cells found in abundance in sputum and bronchoalveolar lavage fluid (BALF) in acute processes are neutrophils. These cells undergo activation with degranulation and release of proteolytic enzymes, which is considered the main factor responsible for damage to the lung tissue (ADAGE et al., 2015).

About the pulmonary defense mechanisms and the pathogenesis of the lesions, especially in systemic diseases such as visceral leishmaniasis (VL), there are few reported in the literature. Histopathological changes in both the domestic dog as in man are of chronic inflammatory nature. In fact, the first reports of interstitial pneumonitis associated with human VL are dated of 1953, characterized by a thickening of the alveolar wall and the presence of mononuclear cell infiltration, especially macrophages (DUARTE et al. 1986; GONCALVES et al., 2003; SILVA et al., 2007).

Given the need for research to seek new therapeutic approaches to minimize the sequelae caused by VL, cell therapy may be a promising alternative in lung tissue repair. Studies with mononuclear stem cells obtained from bone marrow and umbilical cord blood suggest that they can also differentiate into various cell types present in the adult organism (PITTENGER et al., 1999; DUFFIELD; BONVENTRE, 2005). Studies show the greatest plasticity of stem cells from bone marrow, giving them the ability to develop into specific tissues such as the lung tissue and other (POULSON et al., 2002; GHARAEE-KERMANI et al., 2007; LOEBINGER and JANES 2007).

In the case of canine VL, the most affected organs are the kidney and lung and to assess important cellular abnormalities in the lung parenchyma can be used the BALF collection technique as a tool for lung microenvironment analysis and cell populations involved.

Therefore we use a simplified technique of BAL collection, whose aim was to evaluate qualitatively and quantitatively this cell population in the BALF of dogs naturally infected by *Leishmania infantum chagasi* and compare the cellular characteristics of this fluid in positive animals naturally to VL after infusion of bone marrow mononuclear cells (BMMC).

MATERIAL AND METHODS

All procedures were guided by ethical principles established by the National Council for Animal Experiments Control - CONCEA and in accordance with national legislation (Law No. 11.794, of August 8, 2008 and Law No. 9605 of February 12, 1998). In addition, all experimental procedures in this study were approved by the Ethics Committee on Animal Experimentation - CEEA of the Federal University of Piauí - UFPI under No. 056/10.

Animals

This study included a total of 13 adult dogs of both sexes, indefinite race and separate into three groups: negative control (NG) with six dogs not infected by *L. infantum chagasi*; positive group (PG) with seven dogs naturally infected by *L. infantum chagasi*; treated group positive (TPG), where the positive group of four dogs were infused intravenously (IV) with homologously BMMC. Three Animals of PG were submitted to two BAL procedure with an interval of 5 months. All animals were from the Zoonosis Management - GEZOON, and the animals positive for leishmaniasis were diagnosed by indirect immunofluorescence assay (IFA) and the parasitological examination in the Animal Health Laboratory - LASAN / UFPI. The animals received bath with insecticide amitraz on arrival at the Federal University of Piauí - UFPI and were maintained throughout the experiment in the kennel, fed with dry commercial feed and water *ad libitum*, besides sunbathing and clinical follow-up daily. All dogs were treated for ehrlichiosis before the start of the experiments.

Anesthetic procedure

The procedure of obtaining the BAL was performed at the Veterinary University Hospital-HVU / UFPI. Before the surgical procedures, the animals were fasted for 12 hours. All animals were anesthetized with acepromazine 0.2% intramuscular (IM) (0.05 mg / kg) as a premedication, followed by induction with propofol IV (4 mg / kg) and intubated with endotracheal tube (cuffed) for the inhalatory anesthesia with isoflurane.

Euthanasia

After collection of the BALF, excluding animals PG and TPG, which were euthanized after the second obtaining fluid, the animals were euthanized according to the following protocol: Premedication with acepromazine (0.2%, 0.05 mg / kg) along with meperidine hydrochloride (4 mg / kg) IM and subsequent intravenous injection of thiopental sodium (25 mg / kg) for coma induction then proceeded to IV application potassium chloride 19% until checking the loss of vital functions.

Bronchoalveolar lavage

To conduct the BAL, the animal were positioned in dorsal recumbence and intubated with an endotracheal tube n° 7.0 and applied to 20 ml of sterile saline solution 0.9%; stimulating the cough reflex in the animal, collected up the BALF and the volume stored in 50 ml polypropylene conical tubes (Falcon ™) and packed in isothermal container with artificial ice to the tests in Laboratory at Department of Morphology (DMOR) of the Health Sciences Center - UFPI. For determining the number of cells in the bronchoalveolar fluid, to each aliquot of 0.9 ml of cell suspension were added 0.1 ml of crystal violet solution at 0.5% in acetic acid at 30%. And then was performed a cell global counting in Neubauer chamber using the center square and differential count between slide and coverslip. Counts were performed under light microscope with a 40x objective.

Collection and separation of BMMC

The animals of positive group who received IV infusion of homologous BMMC were previously subjected to premedication of Ketamine (0.15 mg / kg) IM, subsequently intubated for maintenance of inhalation anesthesia with isoflurane. After the trichotomy and antisepsis of the iliac crest, was performed a bone marrow puncture (5 ml) using heparinized syringe Luer lock. The blood sample was stored on ice and transported to the Histology Sector at DMOR, the bone marrow aspirate was diluted in 0.9% saline (1: 1) and BMMC were separated by density gradient using Ficoll-Paque Plus™. The resulting solution was centrifuged for 20 minutes at 2000 rpm, and the halo formed aspirated and transferred to another polypropylene tube and added to saline solution plus fetal bovine serum (1: 6) to again centrifuge at 2000 rpm for 7 minutes. The "pellet" formed was resuspended with saline and filtered in mesh. The resulting cell suspension was added to saline, centrifuged again and the cell button resuspended in 2.0 ml of saline solution 0.9%. An aliquot of the cell solution was stained with Trypan Blue (1: 1) and the count and cell viability measured using a Neubauer chamber.

Cell Therapy

The animals of TPG received a BMMC homologous infusion adjusted in concentration of 1.0×10^8 cells intravenously using jugular vein catheter. Obtaining and infusion of BMMC were performed in two stages, with an interval of 15 days for each infusion.

Statistical analysis

For statistical analysis of the total count of the number of cells was performed using the Mann-Whitney test for nonparametric analysis with 95% confidence level ($p < 0.05$) and for the other analyzes, we used the Student's t-test ($p < 0.05$) through IBM SPSS Statistics software.

RESULTS AND DISCUSSION

The naturally infected animals showed clinically oligosymptomatic and with at least three of the following signs: skin lesions, onychogryphosis, alopecia, weight loss, keratitis and typical apathy.

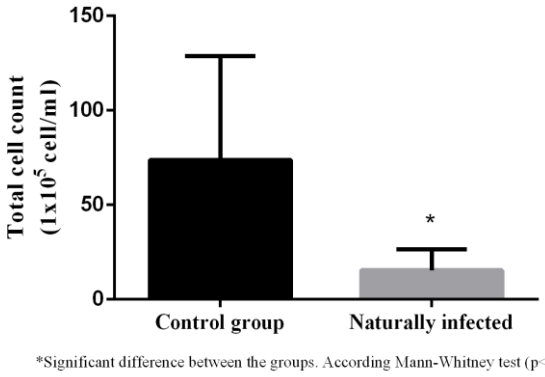
Being the BAL a method invaluable in assessing the inflammatory and immune processes in the lung, verifying the presence of cells on the epithelial surface of the lower respiratory tract (LRT), and to observe the possible occurrence of a new cell population influx into the lungs, the administration of 20 mL saline solution 0.9% in each dog NG, PG and TPG possible to obtain a variable volume fluid for both BAL performed per animal.

The average volume of BALF obtained was 6.0 ml, with variations between 4.0 ml and 10.0 ml. In general, the ratio of the obtained volume was low, considering the volume of injected saline solution, the average yield was 15% of the injected volume, this volume close to that obtained by Mello (2002) Woods et al. (2013) and Woods et al. (2014), but lower than the amount aspirated by Ribas (2010).

The liquid obtained was presented as a colorless, slightly cloudy, with particulate matter and presence of surfactant, appropriate macroscopic characteristics for the collected material (LESSA, 2005; RIBAS, 2010). The presence of surfactant in the fluid is verified by the presence of foam in the aspirate collection tube, showing

that the obtained fluid was withdrawn from LRT (HAWKINS; DeNICOLA; KUEHN, 1990; FINKE 2013).

According to total cell number count found in the BAL, there was a significant difference in the mean of this count between the control animals and naturally infected with *Leishmania infantum chagasi* (Figure 01), where the group of non-infected dogs was observed a greater number of cells / ml, while the total cell counts in dogs with VL was a reflex of immunosuppressed state of them.



*Significant difference between the groups. According Mann-Whitney test (p<0,05).

FIGURE 01: Comparison of total cell count obtained from bronchoalveolar lavage fluid (BALF) of dogs naturally infected with *Leishmania infantum chagasi* (N = 7) and the control group (N = 6).

The largest amount in total number of cells in animals of NG could suggest a reflection of previous poor health conditions of the dogs, possibly still presented some disorder of the respiratory tract, as seen by Woods et al. (2014), who performed the count in BALF cells obtained from animals with diseases of the respiratory tract and observed in one of the samples the count of 14,900 cells / µl. Most animals coming of GEZOON were stray dogs and were confined with several other animals with unknown health condition in the headquarters of management.

In contrast, NG dogs showed no signs and symptoms that reveal any respiratory disease and also evidence of infection by white cell counts made during the experimental period (data not shown). Therefore, based on clinical evidence and literature data, the average value of the total number of cells obtained from NG dogs BALF can be considered as the limits for physiological parameters.

Within the group of naturally infected animals, three animals were used for obtaining the BALF twice with an interval of five months between the two samples. Table 1 shows the total number of cells present in the fluid between the two collections and the second BAL there was a decrease individually in total cell number by up to 95%. However, the average total number of cells between both BAL showed that statistically there was no significant difference and had a high standard deviation value between the values measured for each animal.

Our data showed no significant difference in average total number of cells between the two procedures, could be noted, after a period of five months, the evolution of VL can be observed biologically by degree of immunosuppression of pulmonary immune response of each animals by checking a decrease of more than 50% of the cell population (Table 01).

GROUP		1st BAL Total cell count (1x10^{5}cells /mL)	Kennel time (months)	2nd BAL Total cell count (1x10^{5}cells /mL)	Difference between the 1st and 2nd BAL (%)
Naturally infected animals	Animal 1	19,5	5	1,0	-95%
	Animal 2	28,5	5	4,1	-86%
	Animal 3	1,8	5	0,6	-67%
Mean		16,6[a]	-	1,9[a]	-82,6%

* Means followed by different letters in the same line are significantly different (p <0.05) by Student's t-test

TABLE 1: Total cell count obtained from the BALF of dogs naturally infected with *Leishmania infantum chagasi*, a comparison between the 1st and 2nd BAL.

On the other hand, animals infected naturally by *Leishmania infantum chagasi* and were infused twice with BMMC presented an increase in the number of total cells found in the second BAL (Table 02), suggesting that much of the increase in this cell population was due to BMMC infusion and that they went through the pulmonary microcirculation, since the lung is the first organ encountered by BMMC after infusion.

Gharaee-Kermani et al. (2007) consider the use of stem cells provides new approaches for lung diseases, particularly cells obtained from bone marrow, checking the recruitment of these cells to the injury sites. Based on this statement, it would be fair to say that the data we obtained in the count of cells present in the fluid of the second BAL (Table 02) suggest that BMMC infused reach the lung parenchyma, and this cell kinetics by hematogenous route of vital importance to institute new approaches to cell therapy to assist in the treatment of respiratory disorders of LRT.

In differential analysis of cell type present in the BALF (Table 03) revealed that in all dogs naturally infected there was a significant increase in the number of mononuclear leukocytes after the second BMMC infusion when compared with measured values before the BMMC infusion, indicating the passage of these by the lower portions of the respiratory tract. The low number of lymphocytes in the first BAL corroborates the number found by Barçante et al. (2008). This increased lymphocytes in BALF is considered unusual (HEIKKILA, 2011),

ANIMALS	1st BAL TCC of pre BMMC infusion ($1x10^5$cells/ mL)	2nd BAL TCC of post BMMC infusion ($1x10^5$ cells / ml)	Increased cell percentage in BAL after the 2nd BMMC infusion (%)
F1	6,1	194,0	3080%
M1	19,0	30,0	58%
M2	4,9	190,0	3801%
M3	28,1	31,0	10%
Mean	$14,5^a$	$111,3^a$	666%

* Means followed by different letters in the same line are significantly different (p <0.05) by Student's t-test. ** TCC: Total cell count

TABLE 2: Total cell count obtained from the BAL fluid of dogs naturally infected by *Leishmania infantum chagasi* and infused with BMMC, as scores between the 1st and 2nd BAL.

and in humans is generally associated with non-specific interstitial pneumonia and granulomatous diseases (AMERICAN THORACIC SOCIETY and EUROPEAN RESPIRATORY SOCIETY, 2000). In our study this increase can be strongly associated with higher inflow of similar cells to lymphocytes to the lungs due to the infusion of BMMC and these BMMC can be either hematopoietic origin and mesenchymal.

In contrast, the macrophage differential counts in dogs naturally infected pre and post infusion of BMMC (Table 03) observed to be similar to values found in healthy dogs (BARÇANTE, 2008), even if the resident macrophages are the predominant cell population found in BALF (HAWKINS, 1990; ANDREASEN, 2003; RIBAS, 2010; FINKE, 2013) It is known that the BMMC can also increase the number of macrophages, from the origin of circulating monocytes can differentiate into macrophages (HUSSELL and BELL, 2014), which would explain the numerical increase of over 40% in the differential count in two animals (2/4) of TPG.

It was also possible to observe that the count of polymorphonuclear cells (PMN) present in the BALF declined during the trial period, showing a predominance of mononuclear cells (MN), which characterize chronic

ANIMALS	PMN		Difference between the 1st and 2nd BAL (%)	Total cells MN		Difference between the 1st and 2nd BAL (%)	Macrophage		Lymphocyte	
	1^a	2^a		1^a	2^a		1^a	2^a	1^a	2^a
F1	19	2	-89%	81	98	21%	74	54	7	44
M1	52	1	-98%	56	99	77%	53	79	3	20
M2	51	2	-96%	49	98	100%	43	60	6	38
M3	15	0	-100%	85	100	18%	85	67	0	33
Mean	$34,3^a$	$1,3^b$	-96%	$63,8^a$	65^b	46%	$67,8^a$	$98,8^a$	4^a	$33,8^b$

* Means followed by different letters in the same line are significantly different (p <0.05) by Student's t-test ** PMN: Polymorphonuclear cells; MN: Mononuclear cells; F: Female; M: Male.

TABLE 3: Differential cell count obtained from BALF of dogs naturally infected by *Leishmania infantum chagasi* obtained pre and post 1st infusion and the 2nd BMMC infusion.

inflammatory processes and tissue repair, since neutrophils do not are able to survive long in the inflammatory focus (AMERICAN THORACIC SOCIETY and EUROPEAN RESPIRATORY SOCIETY, 2000). However, in our study, this significant increase in MN cells after BMMC infusion in animals infected by *Leishmania infantum chagasi* reinforces the thesis that after infusion, the migration kinetics of BMMC by hematogenic pathway culminates in its passage the microcirculation and subsequent staging lung parenchyma.

CONCLUSION

The BMMC infusion in animals infected with *Leishmania infantum chagasi* was detected in BALF with a significant increase of MN cells. The BALF analysis revealed that the immune response in the lung is significantly depressed VL and MN cell population rises after infusion of BMMC.

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