THE RELEVANCE OF NUTRITIONAL STATUS AND HISTOPATHOLOGICAL FINDINGS ON THE INFECTIOUS PROCESS OF BALB/C MICE INOCULATED WITH Lacazia loboi

Adriana Sierra Assencio Almeida BARBOSA(1), Suzana Madeira DIÓRIO(2), Silvia Cristina Barboza PEDRINI(3), Adauto José Ferreira NUNES(4), Andréa de Faria Fernandes BELONE(4), Sônia Maria Uso Ruiz SILVA(2), Beatriz Gomes Carreira SARTORI(1), Sueli Aparecida CALVI(5), Fátima Regina VILANI-MORENO(1) & Paulo Câmara Marques PEREIRA(5)

SUMMARY

The aim of this study was to evaluate the effects of the protein-calorie malnutrition in BALB/c isogenic mice infected with Lacazia loboi, employing nutritional and histopathological parameters. Four groups were composed: G1: inoculated with restricted diet, G2: not inoculated with restricted diet, G3: inoculated with regular diet, G4: not inoculated with regular diet. Once malnutrition had been imposed, the animals were inoculated intradermally in the footpad and after four months, were sacrificed for the excision of the footpad, liver and spleen. The infection did not exert great influence on the body weight of the mice. The weight of the liver and spleen showed reduction in the undernourished groups when compared to the nourished groups. The macroscopic lesions, viability index and total number of fungi found in the footpads of the infected mice were increased in G3 when compared to G1. Regarding the histopathological analysis of the footpad, a global cellularity increase in the composition of the granuloma was observed in G3 when compared to G1, with large numbers of macrophages and multinucleated giant cells, discrete numbers of lymphocytes were present in G3 and an increase was observed in G1. The results suggest that there is considerable interaction between Jorge Lobo’s disease and nutrition.

KEYWORDS: Jorge Lobo’s disease; Lacazia loboi; Malnutrition; Mice; Histopathology.

INTRODUCTION

The interaction between nutrition and infection has been considered one of the major problems for the development and survival of humans. Many studies evaluating the effect of malnutrition on mice infected with Toxoplasma gondii, Schistosoma mansoni, Leishmania chagasi, Staphylococcus aureus and Mycobacterium tuberculosis, however, there aren’t studies relating nutritional aspects to the Jorge Lobo’s disease so far.

Jorge Lobo’s disease, also known as lobomycosis or lacaziosis, is a chronic, granulomatous, cutaneous-subcutaneous, fungal infection caused by the fungus Lacazia loboi (L. loboi) and is characterized by isolated or multiple coalescing lesions that can occur clinically in a localized or disseminated form.

In 2007, a study by BRITO et al. related 318 diagnosed cases of such disease in Brazil, predominantly in the Amazon region, with a total of 490 cases estimated worldwide. In 2010, a study by WOODS et al. described 249 patients who were attended to the Department of Sanitary Dermatology, Rio Branco, Acre, Brazil, between 1998 and 2008. The number of patients is relatively small, but the real situation of the disease as a public health problem is not known, because Jorge Lobo’s disease is not a disease of compulsory notification.

This mycosis is more common in males due to their occupational activities, and is generally manifested in people aged between twenty and forty-one, coinciding with their work-related activities, when they are naturally exposed to the pathogen.

Histopathologically, this mycosis is characterized by an intense and diffuse histiocytic reaction, often with fibrosis, restricted to the dermis, consisting of large numbers of multinucleated giant cells (MGC), both foreign-body-type and Langhans-type, and numerous fungi mainly inside these cells. Plasma cells, neutrophils and a small number of lymphocytes are also presented.

An immunohistochemical study conducted by VILANI-MORENO et al. showed that inflammatory cell infiltrates presented the following frequency order: macrophages CD68 > T lymphocytes CD3 > T lymphocytes CD4 > T lymphocytes CD8 > Natural Killer cells CD57 > Plasma cells CD79 > B lymphocytes CD20. According to the authors, the large amount of fungi in the lesions, the disorganized granuloma and the changes in the profile of the Th2 cell cytokine pattern, suggest that...
patients showing immunoregulatory disorders with the mycosis might be responsible for the lack of containment of the pathogen.

The fungus *L. loboi* has not been grown in artificial culture media so far; however, MADEIRA et al.\(^3\) developed an experimental model for the study of Jorge Lobo’s disease, by inoculating the fungi obtained from patient’s skin lesions, intradermally, into the footpads of BALB/c strain mice. These authors found that eight months post-inoculation, the footpad of the mice showed macroscopic lesions and histological changes, similar to those found in human disease, with a large number of viable fungi.

A more recent study has shown the experimental reproduction of Jorge Lobo’s disease in BALB/c mice inoculated with fungi obtained from previously infected mice presenting macroscopic lesions. Hence, BALB/c mice is considered an excellent mouse strain for the maintenance of *L. loboi* in the laboratory\(^2\).

Considering this, as there had been no other studies on the role of nutritional status in Jorge Lobo’s disease, the aim of the present study was to evaluate the effects of the protein-calorie malnutrition (PCM) in BALB/c isogenic mice infected with *L. loboi*, by determining the weight of their bodies and organs, the viability index and the total number of fungi in the footpad, and also the histopathological analysis of organs and footpad.

**MATERIALS AND METHODS**

**Experimental groups:** A total of 60 isogenic 12-week-old male BALB/c mice were kept at the Lauro Souza Lima Institute. All animals were housed in plastic cages (five mice per cage) with white wood chips for bedding and access to commercial food Nuvilab CR-1 (Nuvital, Colombo, PR, Brazil), which is specifically made for the feeding of laboratory rodents, drinking water, under controlled lighting (12-h day/12-h night cycle) and temperature (22 ± 2 °C) conditions. The animals were distributed into four groups: (G1) 15 mice inoculated with the fungus and under dietary restriction, (G2) 15 mice not inoculated with the fungus and under dietary restriction, (G3) 15 mice inoculated with the fungus and on a normal diet and (G4) 15 mice not inoculated with the fungus and on normal diet. The research project was approved by the Ethics Committee on Animal Experiments of the Butocatu School of Medicine, UNESP.

**Fungal suspension:** *L. loboi* was obtained from the footpads of BALB/c mice previously inoculated for maintenance of the strain\(^2\). The animals were sacrificed and the footpads were removed and macerated in 0.9% sterile saline. The fungal suspension (pool) obtained was evaluated with respect to the number of fungi and its viability was determined by vital staining with fluorescein diacetate-ethidium bromide, as described for *L. loboi* by VILANI-MORENO & OPROMOLLA\(^1\). The strain used in our experiments was molecularly characterized according to VILELA et al.\(^2\).

**Inoculation:** Mice were inoculated intradermally in both hind footpads with 0.03 mL of fungal suspension containing 1.4 x 10^6 fungi, with inoculum concentration of 4.8 x 10^7/mL and viability index of 38%.

**Diet and weight of the animals:** Before the start of the study, all animals were weighed; the animals in groups G1 and G2 were submitted, for 20 days, to a restricted diet with a daily offer of 80% of the quantities ingested by groups G3 and G4. The dietary restriction was conducted in order to achieve a weight loss of about 20%. Twenty days after receiving a normal diet or dietary restriction, the animals were infected. After the inoculation, the animals of groups G1 and G2 remained on dietary restriction until the period they had to be sacrificed, at the final moment. All groups of animals were weighed monthly. The food and animals were weighed on a digital scale with 0.25g to 5,000g precision (model AS5500C Marconi, Piracicaba, SP, Brazil).

**Euthanasia of the animals:** Four months after the inoculation (period necessary for the appearance of the macroscopic lesions in the footpads, according to BELONE et al.\(^3\)), the animals were euthanized using carbon dioxide (CO\(_2\)). The liver and spleen were excised and weighed on a digital scale with 0.01g to 210g precision (model Metler Toledo AB204, Barueri, SP, Brazil) and the material underwent histopathological evaluation. The footpads were excised to determine the viability index and the total number of fungi, and also to perform the histopathology.

**Histological sections of organs and footpads:** The footpads and organs were fixed in 10% buffered formalin for 24 hours, and then prepared to be embedded in paraffin, according to the usual technique. Histological sections of 4 μm thick were stained with Hematoxylin-Eosin (HE) and Gomori’s methenamine silver (GMS)\(^9\).

**Histopathological evaluation:** The slides of the HE-stained organs underwent histopathological evaluation and the GMS-stained slides were examined for the presence of fungi in the organs. The HE-stained footpad slides were used to examine the characteristics of dermal inflammatory infiltrate as well as cell elements: macrophages, lymphocytes, neutrophils, plasma cells and MGC were quantified on a semi-quantitative 0-4 + scale where: 0 = absent, 1 = minimal, 2 = mild, 3 = moderate, 4 = intense\(^17\,^30\). The GMS-stained slides were examined for the presence of fungi in the footpads, and the findings were semi-quantified on a 0 - 4 + scale as described above.

**Statistical Analysis:** All data were analyzed with the SAS for Windows software, version 9.2. The Tukey test was performed for the analysis of the following variables: body weight, liver, spleen and fungi viability index. Poisson or negative binomial distributions were used for the analysis of the number of fungi, followed by the Likelihood-ratio test. In all cases, the significance level was set at 5%.

**RESULTS**

Regarding animal weight, at the first moment, no significant differences were observed among the studied groups. Then, the animals in groups G1 and G2 were submitted to dietary restriction, while the animals in groups G3 and G4 received a normal diet. At the time of inoculation, a significant decrease was observed in the weight of the animals in groups G1 and G2 and a significant increase in weight was observed in the animals from groups G3 and G4, when compared to the initial conditions of each group (*p* < 0.05). After the 4-month period, there were no significant differences in the weight of the animals from groups G1 and G2. There was a significant increase, however, in the weight of the animals from groups G3 and G4, when compared to the moment of inoculation of each group (*p* < 0.05) (Table 1).
There has been a significant interaction between the effect of the diet and the studied groups, as the weight of the liver and spleen of the malnourished groups G1 and G2 showed significantly lower values when compared to the groups with no dietary restrictions G3 and G4 ($p < 0.05$) (Table 1).

The histopathological analysis of the liver and spleen showed no differences among the studied groups. In accordance with the weight of the organs, histological sections of the liver revealed atrophic hepatocyte trabeculae in the malnourished groups (G1 and G2), while in both groups inoculated with the fungus *L. loboi* (G1 and G3), the liver showed vacuolar degeneration and more intense nuclear reactivity when compared to the non-inoculated groups. Histological sections of the spleen revealed only sinusoidal congestion in all groups. The study of fungi, through histopathological analysis, revealed that GMS-stained sections of the liver and spleen, in the inoculated groups (G1 and G3), showed no dissemination of fungi to these organs.

The footpads of the animals of the two groups (G1 and G3) showed macroscopic lesions, which in general were higher in group G3 (inoculated) when compared to group G1 (Fig. 1A and 1B).

The total number of fungi recovered from the footpads of the infected animals (G1 and G3) was $1.95 \times 10^9$/mL in G1 and $3.22 \times 10^9$/mL in G3 ($p < 0.05$). The viability index of *L. loboi* was higher in G3 animals ($41.8\%$) when compared to G1 animals ($32.2\%$) ($p < 0.05$).

The histopathological study of the footpads of the mice inoculated with *L. loboi*, G1 and G3, irrespective of the PCM, showed similar morphological patterns. We observed the presence of inflammatory infiltrate predominantly composed by macrophages (4+) and MGC, both foreign body-type and Langhans-type, and numerous fungi mainly inside these cells, many of them with morphological characteristics of viability (Fig. 1C and 1D).

In group G3, lymphocytes were present in discrete numbers (2+) and were dispersed between macrophages or forming small clumps around blood vessels, macrophages and MGC. Neutrophils were present in moderate numbers (3+), also disperse in the infiltrate or aggregated forming microabscesses. Scattered areas with more lymphocytes or neutrophils were observed, although the neutrophils were always present in greater numbers (Fig. 1D and Fig. 2).

In group G1, the inflammatory infiltrate was less intense in comparison to that of group G3. However, these animals showed an increase in the number of lymphocytes (3+), while their number of neutrophils did not change (3+). Cell distribution was similar in both groups G1 and G3, and rare plasma cells were observed (Fig. 1C and Fig. 2).

**DISCUSSION**

Jorge Lobo’s disease is a rare mycosis that occurs predominantly in the Brazilian Amazon region. Unknown factors are related to the
The study of COUTO et al.5, about changes on the liver function in mice with schistosomiasis associated with malnutrition showed that both infection and/or malnutrition interfered in the levels of hepatic biochemical indicators, but the most important changes in liver function occurred during the intense inflammatory process caused by schistosomiasis, leading to increased liver enzymes.

Regarding the histopathological study of the liver, studies performed by OLIVEIRA et al.16 and COUTINHO et al.3 showed that malnutrition affects several regenerative mechanisms, such as decreased fibroblast proliferation, and synthesis of collagen and albumin. They also showed that the nutritional status of the host plays an important role in changing the connective tissues in murine hepatic schistosomiasis.

Another important aspect to be highlighted is the appearance of macroscopic lesions on the footpad that were smaller in malnourished animals (G1) when compared to the nourished group (G3). A less intense inflammatory reaction was also observed in the nourished group. The viability index and total number of fungi found in the footpads of the inoculated mice showed an increase in the nourished mice when compared to the malnourished mice. As there are no other studies linking malnutrition and Jorge Lobo’s disease, the comparisons of our results are restricted to the inoculated mice with no dietary restriction.

Studies by MADEIRA et al.14 with BALB/c mice, and VILANI-MORENO & OPROMOLLA13 in cutaneous lesions of humans, showed viability indexes similar to those found in our study. Also, a research conducted by BELONE et al.2 with mice inoculated with L. loboi showed that the number of fungi recovered at four months after inoculation was similar to ours.

Regarding the histopathological analysis of footpads, increased global cellularity in the composition of granuloma in the nourished group was observed in comparison to that of the malnourished group, in addition to the proportional increase of lymphocytes in the malnourished group. Studies with malnourished mice inoculated with Staphylococcus aureus and Mycobacterium tuberculosis3,10 showed a decrease in the number of peripheral blood lymphocytes, suggesting that these cells may be migrating to the site of infection, similar to the results found in our study.

These findings are important and lead to the hypothesis that malnutrition in Jorge Lobo’s disease can cause certain resistance to fungal growth, characterized by the lower viability index, total number of fungi and reduced size of footpads found in the malnourished group. This fact is unexpected because malnutrition has been most commonly associated with increased susceptibility to infectious agents12,22. However, this finding could be explained by the competition for nutrients between the host and the pathogen, as described in other studies22,23.

Similar results were reported by FRANÇA et al.4 in an experimental study with BALB/c mice inoculated with Staphylococcus aureus using the same model of malnutrition as ours. This study showed that malnourished mice had a smaller amount of bacteria in the lungs compared to the nourished mice.

The study of OARADA et al.30 with BALB/c mice inoculated with Paracoccidioides brasiliensis (P. brasiliensis) using diet of 1.5% casein showed higher antifungal activity in the liver and spleen when compared...
with the 20% casein diet, suggesting that protein restriction led to increased host resistance to P. brasiliensis. In another study, OARADA et al.9 found a greater number of fungi in the liver and spleen when using a diet of 54% protein, when compared to a 5 and 20% casein diet. According to the authors, the results suggest that a higher protein diet reduces host resistance to P. brasiliensis. It’s an interesting finding since P. brasiliensis and L. loboi have many similarities. A survey of the phylogenetic analysis of L. loboi held by HERR et al.7 suggested that L. loboi is a dimorphic fungus, taxonomically related to P. brasiliensis and that they belong to the Onygenales order, Ajellomycetaceae family, which could explain the similarity of results in our study.

When all the results are analyzed in conjunction, one important conclusion is that there is an interaction between Jorge Lobo’s disease and nutrition, and as most patients have low socioeconomic conditions and are likely to present nutritional deficiency, new studies are needed to clarify the mechanisms involved in this interaction, especially considering that in humans the disease may develop in localized and disseminated clinical forms.

RESUMO

A relevância do estado nutricional e alterações histopatológicas no processo infeccioso de camundongos BALB/c inoculados com Lacazia loboi

O objetivo do estudo foi avaliar os efeitos da desnutrição proteico-calórica em camundongos isogênicos da linhagem BALB/c inoculados com Lacazia loboi, empregando parâmetros nutricionais e histopatológicos. Foram constituídos quatro grupos: G1- inoculados com restrição dietética; G2- não inoculados com restrição dietética; G3- inoculados sem restrição dietética; G4- não inoculados sem restrição dietética. Após instalada a desnutrição, os animais foram inoculados via intradérmica no coxim plantar e após quatro meses foram sacrificados para remoção do coxim plantar, fígado e baço. A infecção não exerceu grande influência no peso corporal dos camundongos. O peso do fígado e baço apresentou redução nos grupos desnutridos em comparação aos grupos nutridos. A lesão maculopapular, a viabilidade e o número total de fungos dos coxins plantares dos camundongos inoculados revelaram aumento no G3 quando comparado com o G1. Em relação à análise histopatológica dos coxins plantares observou-se aumento da celularidade global na composição do granuloma no G3 em relação ao G1, com grande número de macrófagos e células gigantes multinucleadas, discretos números de linfócitos estavam presentes em G3 e aumentados no G1. Os resultados sugerem que existe grande interação entre nutrição e doença de Jorge Lobo.

CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

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