http://dx.doi.org/10.1590/1678-4162-12663

Arq. Bras. Med. Vet. Zootec., v.75, n.6, p.1061-1064, 2023

Evaluation of genome stability of bone marrow cells of dogs with naturally infected visceral leishmaniasis

[Avaliação da estabilidade genômica de células da medula óssea de cães com leishmaniose visceral naturalmente infectados]

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ABSTRACT

The aim of this article is to identify and evaluate the genomic stability of visceral leishmaniasis in naturally infected dogs. A total of 32 dogs participated in the study, 24 of which were asymptomatic animals, naturally infected, and 8 uninfected, used as a control group. The comet and micronucleus assay tests were performed on bone marrow cells obtained by means of aspiration puncture. For data analysis, the Shapiro-Wilk test was used to verify the normality of the data, and then the Mann-Whitney and T-tests for the comparison between the infected and control groups. The analysis of samples from naturally infected animals and healthy animals showed that the infection caused by the protozoan responsible for leishmaniasis was not able to induce DNA damage in the cells of the infected animals. In the results obtained through the micronucleus test, an increase in the number of micronuclei were observed in polychromatophilic erythrocytes in the medullary tissue of the infected group, when compared to the control group. As a main contribution, the results expressed in this study, consider that the comet and micronucleus assays are suitable for the biomonitoring of genomic stability in bone marrow cells of naturally infected dogs with visceral leishmaniasis.

Keywords: comet assay, genotoxicity, leishmaniasis, micronucleus test

RESUMO

O objetivo deste artigo é identificar e avaliar a estabilidade genômica em células da medula óssea de cães com leishmaniose visceral naturalmente infectados. Um total de 32 cães participaram do estudo, sendo 24 animais assintomáticos, infectados naturalmente, e oito não infectados, utilizados como grupo controle. Os testes de ensaio de cometa e micronúcleo foram realizados em células da medula óssea obtidas por meio de punção aspirativa. Para análise dos dados, o teste de Shapiro- Wilk foi empregado para verificar a normalidade dos dados, e, em seguida, o teste de Mann-Whitney e o teste t para a comparação entre os grupos infectado e controle. A análise das amostras dos animais naturalmente infectados e dos animais saudáveis demonstrou que a infecção causada pelo protozoário responsável pela leishmaniose não foi capaz de induzir danos ao DNA das células dos animais infectados. Já nos resultados obtidos por meio do teste do micronúcleo, foi observado um aumento no número de micronúcleo em eritrócitos policromatofílicos no tecido medular do grupo infectado, quando comparado ao grupo controle, confirmando, assim, o potencial mutagênico da leishmaniose visceral em cães infectados naturalmente.

Palavras-chaves: ensaio de cometa, genotoxicidade, leishmaniose, teste de micronúcleo

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Sumitted: January 13, 2022. Accepted: March 7, 2023.

INTRODUCTION

Visceral leishmaniasis (VL) is one of the most neglected tropical zoonoses in the world. The disease is mainly caused by the protozoan Leishmania infantum and transmitted by infected female phlebotomies (Haldar *et al*., 2011) and has high incidence rates in the Americas, with 96% of cases occurring in Brazil (Opas, 2019).

The dog is considered, in the urban transmission cycle, the main reservoir, through which human beings can become infected (Albuquerque *et al*., 2017). In this species, infection by *Leishmania infantum* triggers a variety of responses that can range from asymptomatic infection to the development of active disease (Solano-Gallego, *et al*. 2011; Brasil, 2016).

L. infantum causes suppression of cell-mediated immunity, enabling the uncontrolled dissemination and multiplication of the protozoan (Brasil, 2014). The presence of amastigotes in the tissue increases the production of nitric oxide by macrophages and causes genotoxic changes (Oliveira *et al*., 2011). This mechanism of inducing DNA damage has been described in leukocytes from mice infected with *L. infantum* (Oliveira *et al*. 2011; Moreira *et al*., 2017). However, these genotoxic effects have not yet been described in dogs with LV.

Thus, this work aims to evaluate the genomic stability in bone marrow cells from naturally infected dogs with visceral leishmaniasis.

MATERIAL AND METHODS

This work was approved by the Ethics Committee on the Use of Animals (CEUA / UFMS) under protocol number 1018 / 2019.

Convenience sampling included 32 dogs of both sexes, without breed restriction and aged between 1 and 8 years. Of these, 24 were asymptomatic animals with visceral leishmaniasis, diagnosed by immunochromatographic test (DPP® LVC Bio-Manguinhos) and parasitologically confirmed, delivered to the Campo Grande-MS Zoonosis Control Center for euthanasia, following the Interministerial Ordinance number 1426, of 11 of July 2008.

Considering the frequency of coinfections in the region, all animals were submitted to the Polymerase Chain Reaction (PCR) test for *Babesia canis* and *Erhlichia sp*. and positive animals were excluded from the study. Campo Grande is in a non-endemic area for *Trypanosoma cruzi* and *Dirofilaria immitis*, therefore infections for these agents have not been investigated. The control group consisted of 8 dogs without clinical changes and negative for the same tests performed on infected animals.

For the analyses, 2ml of medullary blood were collected by puncture of the proximal humerus, with the aid of a Jamshidi needle, following the methods recommended by Grindem *et al*. (2009). The sampled material was used to make individual slides for the comet and micronucleus tests, following the methodology described below for each of the analyses.

A 20µL sample, at a 1:400 dilution, of the medullary cells with saline solution (approximately 20,000 nucleated cells) was used for the comet assay, which was performed according to Oliveira *et al*. (2013). To assess DNA damage, the parameters Tail Length, percentage of DNA in the tail (%DNA) and Tail Moment were calculated.

To assess the existence of micronuclei, the method described by Oliveira *et al*. (2009). A total of 2000 erythrocytes from each sample were smoothed. Micronuclei were identified by strong green-yellow fluorescence.

To develop descriptive statistics, medians and standard deviations were calculated for both analyses. Data were tested for normality using the Shapiro-Wilk test. The Mann-Whitney test was used to compare the control and infected groups, with a significance level of 0.05.

RESULTS

The results of the comet assay and micronucleus test are shown in Table 1.

The comet assay did not recognize significant variations in the percentage of DNA in the tail ($p=0.17$), length of the comets ($p=0.07$) or in the moment of the tail ($p=0.39$), in the analyzed cells, when we compared the control group to

dogs with LV, ruling out possible DNA damage in these animals.

In the investigation of chromosomal damage by the micronucleus test, it detected an increase in the number of micronuclei in polychromatic erythrocytes (MNEPC) indicating a high correlation with the risk of carcinogenesis.

Table 1. Median, standard deviation and statistical difference in comet and micronucleus assay tests performed on medullary cells from dogs with visceral leishmaniasis and from the control group

	Control Median (SD)	Infected Median (SD)	*p-value*
(%) DNA in the tail	9.64±4.35	11.34±3.77	0.17
comet length	29.5±4.18	33.77±5.86	0.07
Tail Moment	0.60±0.51	0.59±0.30	0.39
MNEPC	15±5.17	79±6.1	0.0004*

SD - Standard deviation, MNEPC - micronucleus in polychromatophilic erythrocytes, * indicates statistical difference by Mann-Whitney test.

DISCUSSION

The comet assay refers to a useful and widely used technique for evaluating DNA damage and repair in individual cells (Brianezi *et al*., 2009), in this study the comet assay was used to identify the genomic instability that the protozoan responsible for VL can cause cells in naturally infected dogs.

The results obtained by the comet test showed that the action of this protozoan was not able to induce damage to the DNA of cells from naturally infected dogs, not corroborating the study by Moreira *et al*. (2017), which confirmed genotoxic alterations in cells from mice experimentally infected with *L. infantum.*

Considering that the comet assay can detect genomic lesions, the micronucleus test was carried out in a complementary way to determine if the oxidative lesions would be fixed by DNA repair mechanisms.

The results obtained using the MN test showed that *Leishmania* infection was able to increase the frequency of micronucleated cells (p 0.0004) compared to the negative control, indicating permanent damage to erythrocytes and the cytotoxic potential of the agent in the bone marrow of animals, confirming the observation of Moreira *et al*. (2017, in mice experimentally infected with *Leishmania infantum.*

Fenech *et al*., 2011; Matzenbacher *et al*., 2017, state that the presence of micronuclei can be considered a biomarker for the detection of chromosomal aberrations induced in cells that have undergone the action of different agents.

According to Heuser *et al*. (2008); Araldi *et al*. (2015) and OECD (2016) the comet assay test and the micronucleus test have the robustness, sensitivity and statistical power necessary to assess and identify genotoxicity. Therefore, the results obtained in the present work confirm the genotoxicity, in the bone marrow cells of naturally infected dogs, induced by the infection by *L. infantum.*

In summary, this is the first study to investigate the ability of the LVC agent to promote genotoxic changes in bone marrow cells from naturally infected dogs. Furthermore, this study points out that micronucleus tests and comet assay contributed as low-cost biomarkers in the analysis of these effects that can be recommended in future studies, associated with diagnostic tests in the investigation of the effects of LV in dogs.

CONCLUSION

The increased frequency of micronucleated cells identified using genomic stability biomarkers confirmed the mutagenic potential of visceral leishmaniasis in naturally infected dogs.

ACKNOWLEDGMENTS

This study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) - Finance Code 001, the Federal University of Mato Grosso do Sul (UFMS).

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