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Ascorbic acid reduces the genetic damage caused by miltefosine (hexadecylphosphocholine) in animals infected by *Leishmania (Leishamnia) infantum* without decreasing its antileishmanial activity

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ABSTRACT

Leishamaniasis is a neglected disease caused by over 20 *Leishmania* species, occurring in more than a hundred countries. Miltefosine (hexadecylphosphocholine) is the single oral drug used in treatment for leshmaniases, including cases of infections resistant to pentavalent antimony. Our group has recently demonstrated the ability of miltefosine to cause genomic lesions by DNA oxidation. Acknowledging that antioxidant compounds can potentially modulate Reactive Oxygen Species (ROS), our study verified whether ascorbic acid reduces the genotoxic and mutagenic effects caused by miltefosine, and whether it interferes with drug efficacy. For this purpose, uninfected Swiss mice received simultaneous (single dose treatment) miltefosine and ascorbic acid (gavage and intraperitoneally), besides pre and post treatments (ascorbic acid 24 h before and after drug administration); furthermore, Balb/c mice infected with *Leishmania infantum* received miltefosine plus ascorbic acid (repeated doses treatment). We conducted comet assays, micronucleus tests, dosages of superoxide dismutase enzyme and parasitic burden by the limiting dilution assay. We observed that ascorbic acid administered intraperitoneally displayed a protective effect over damage caused by miltefosine. However, this effect was not not observed when the same doses were administered via gavage, possibly due to low serum levels of this antioxidant. Ascorbic acid's protective effect reinforces that miltefosine damages DNA by oxidizing its nitrogenous bases, which is reduced by ascorbic acid due to its ability of protecting genetic material from the action of ROS. Therefore, our results show that this drug is efficient in reducing parasitic burden of *L. infantum*.

1. Introduction

Leishmaniasis comprise a group of non-contagious, highly neglected, parasitic and chronic infectious diseases caused by protozoa of the genus *Leishmania* (Brasil et al., 2017). There are three main forms of the disease: cutaneous leishmaniasis, the most common form, which causes skin lesions; mucocutaneous leishmaniasis, which leads to partial or total destruction of mucous membranes; and visceral leishmaniasis, which is fatal if not treated appropriately. Visceral leishmaniasis is broadly distributed and recorded in 102 countries, with over 90% of the cases occurring in the following countries: Bangladesh, Brazil, Ethiopia, India, South Sudan and Sudan. We estimate that around 700 thousand to 1 million new cases of this disease surface every year, out of which 500 thousand correspond to the visceral form of leishmaniasis, generating 20 to 30 thousand deaths a year (WHO, 2017).

Treatment for leishmaniasis is very restricted, consisting mostly of two pharmacological lines: antimonials (meglumine antimoniate and sodium stibogluconate) and non-antimonials (pentamidine, amphotericin B, paromomycin and miltefosine). Pentavalent antimonials are still commonly used (Almeida and Santos, 2011), even if several studies have already declared them highly toxic (Demicheli et al., 2002; Kato et al., 2014) and mutagenic (Lima et al., 2010; Cantanhêde et al., 2015; Moreira et al., 2017; de Jesus et al., 2018).

In the past decade, a non-antimonial drug has gathered attention in treatment of leishmaniasis: miltefosine (hexadecil 2- (trimethylazionyl) ethyl phosphate). Also known as hexadecylphosphocholine, miltefosine was created in the United States, in the 1980's, originally for treatment of skin cancer. Following studies demonstrated that it also presented antileishmanial activity and, in 2002, countries such as India, Nepal and Bangladesh introduced it in treatment of leishmaniasis (Moore and

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Lockwood, 2010). In 2011, miltefosine (Impavido®) was included in a list of essential medications by the World Health Organization as an antileishmanial drug and is currently the single antileishmanial drug of oral administration (FDA, 2013), prominent for being an alternative in the maintenance of treatment, including cases of infections resistant to conventional therapy with the pentavalent antimonial (Sundar and Chatterjee, 2006).

Recently, our laboratory demonstrated the ability of this antileishmanial to cause apoptosis, necrosis and genomic lesions with oxidation of purine bases in mammalian cells (Castelo Branco et al., 2016). Oxidation of bases occurs due to the oxidative stress caused by the imbalance between the Reactive Oxygen Species (ROS) and the antioxidant defense system, with a predominance of ROS (Vasconcelos et al., 2007).

To control the formation of ROS and other reactive species, cells have an antioxidant defense system classified as enzymatic and non-enzymatic. The enzymatic system is represented mainly by the antioxidant enzymes: superoxide dismutase (SOD), which are metalloproteins that catalyze the dismutation of superoxide (O_2^-) in oxygen (O_2) and hydrogen peroxide (H_2O_2); catalase (CAT) that acts on the decomposition of H_2O_2 into O_2 and H_2O; and glutathione peroxidase (GPx), which acts on peroxides in general (Marín et al., 2006; Vasconcelos et al., 2007). The non-enzymatic antioxidant system consists of several substances, such as glutathione (GSH), the main intracellular antioxidant compound, β-carotene tocopherols (vitamin E), ascorbic acid (vitamin C) and others (Vasconcelos et al., 2007; Nimse and Pal, 2015).

Ascorbic acid is an important antioxidant in the combat against certain types of cancer, cardiovascular diseases and other diseases associated with oxidative stress (Harrison, 2012). Even though humans are unable to synthesize this antioxidant, it participates in several physiological activities, such as: collagen synthesis; ability to recycle vitamin E; promoting cholesterol reduction and resistance to infections by participating in the immunological activity of leukocytes (Cerqueira et al., 2007; Grangeiro et al., 2013). On the other hand, it is also known that ascorbic acid can be toxic in large quantities, instead taking prooxidant action, which actually favors the occurrence of oxidative stress (Halliwell, 2001; Kato et al., 2014).

The antioxidant property of ascorbic acid in eliminating free radicals suggests that this molecule can modulate oxidative damage to DNA in mammalian cells. Thus, this study investigated the ability of ascorbic acid to decrease the genotoxic and mutagenic effects caused by miltefosine in the *in vivo* system, with and without *Leishmania infantum* infection, as well as to verify whether it interferes with the antileishmanial action of the drug.

2. Material and methods

2.1. General study design

To evaluate the capacity of ascorbic acid to protect the DNA damage caused by miltefosine, two strains of mice presenting different genetic backgrounds, Swiss and Balb/c (non-susceptible and susceptible to *Leishmania* infection), were treated with both compounds using single (simultaneous, pre and post treatment) and repeated doses. Thus, the effect of ascorbic acid on DNA damage was assessed under different conditions.

Firstly, considering that miltefosine causes genomic instability and mutations in uninfected Swiss mice (Castelo-Branco et al., 2016), the ability of ascorbic acid to protect the DNA was assessed by comet assay and micronucleus test. For that, Swiss mice received single dose of both compounds in simultaneous, pre and post treatments. After 24 h, peripheral blood was obtained from the tail of each animal to perform the comet assay. Thereafter, the animals were euthanized by anesthetic overdose (Xylazine - 60 mg/kg and Ketamine - 300 mg/kg) and then the femurs were removed to carry out micronucleus test.

Secondly, to evaluate the effects of ascorbic acid in infected animals, Balb/c mice were treated using repeated doses, according to the World Health Organization for human treatment. Toxicogenetic tests were performed by comet and micronucleus assays, and the parasite burden was determined in lymph node and spleen by limiting dilution. Moreover, considering previous data that showed miltefosine causes oxidation of DNA bases (Castelo-Branco et al., 2016), enzyme superoxide dismutase activity was measured in serum. Briefly, on 14th and 28th days after treatments, peripheral blood was obtained from the tail of each animal to perform comet assay. Then, the animals were anesthetized (Xylazine-20 mg/kg and Ketamine-100 mg/kg) to collect bood for the measurement of superoxide dismutase activity. Therefore, the femurs were removed to carry out micronucleus test and, finally, the spleen and lymph node were removed to determine parasite burden by limiting dilution assay.

2.2. Animals

Male Swiss *Mus musculus* mice (n = 5/group) were obtained from the Central Bioterium of the Federal University of Maranhão – UFMA (Maranhão, Brazil) and Balb/c *Mus musculus* mice were obtained from the Bioterium of the University of Campinas (São Paulo, Brazil), all weighting 30 g and aging around 60 days. Animals were kept in polycarbonate boxes under controlled conditions: temperature around 21 ± 2 °C and alternated 12 h light-dark cycle. Our project has been approved by the Animal Experimentation Ethics Committee (CEUA) of UFMA (n° 23115.009229/2014-21).

2.3. Parasites

Promastigotes of L. *infantum* (MHOM/BR/1970/BH46) were cultured in Schneider's medium (Sigma, St Louis, MO, USA), which was supplemented with 10% Heat-Inactivated Fetal Bovine Serum (GIBCO) at 27 °C. To perform the tests, Balb/c mice were infected in the right hind footpad with 10^7 promastigotes in stationary phase of *L. infantum*, as mentioned by de Jesus Pereira et al. (2015) and Martins et al. (2015). The infection was maintained for 28 days (Marques-da-Silva et al., 2005; Serafim et al., 2010) and on the 29th day the antileishmanial treatments were performed.

2.4. Drugs and treatment

Assays with miltefosine were conducted under different treatment conditions. For this purpose, animals were divided into two groups: 1) uninfected Swiss mice receiving a single dose (70 mg/kg) of miltefosine (Chemical Cayman Company, MI, USA, batch 0463792-1) as previously described (Castelo Branco et al., 2016); 2) infected Balb/c mice which received miltefosine (Impavido®) at 2.5 mg/kg for 28 days, as recommended by the World Health Organization for human treatment; and another group that received 5.0 mg/kg for 14 days. In both assays, the drug was dissolved in distilled water and administered via gavage. Miltefosine capsules (batch 1C2130A) were gently conceded by Prof. Dr. Carlos Costa from Federal University of Piauí, Brazil.

To evaluate protective effect of ascorbic acid (Sigma-Aldrich, St. Louis, MO, batch 69085) over DNA damage induced by miltefosine, Swiss mice received single doses (30, 60 and 120 mg/kg) of ascorbic acid simultaneously to the antileishmanial (70 mg/kg). In order to evaluate the effect of the ascorbic acid exposure route, one group of these animals received the antioxidant via gavage (So30, So60 and So120 groups) and another group received via intraperitoneal (Si30, Si60 and Si120 groups). Besides, intermediate dose (60 mg/kg) was administered 24 h before (pre treatment) and 24 h after (post treatment) miltefosine administration. The doses of ascorbic acid were based on previous study (de Jesus et al., 2018). The negative control (NC) group was administered distilled water.

Concerning the effect of ascorbic acid in repeated dose treatment,

Table 1
Experimental groups used to evaluate protective effect of ascorbic acid over genetic damage induced by miltefosine.

Group	Chemical Agents	Doses (respectively)	Exposure route
Swiss (acute treatment)			
NC	distilled water	–	gavage
AA	ascorbic acid	60 mg/kg	gavage
MT	miltefosine	70 mg/kg	gavage
So30	ascorbic acid + miltefosine	30 mg/kg + 70 mg/kg	gavage
So60	ascorbic acid + miltefosine	60 mg/kg + 70 mg/kg	gavage
So120	ascorbic acid + miltefosine	120 mg/kg + 70 mg/kg	gavage
PRE	ascorbic acid (24 h before) + miltefosine	60 mg/kg + 70 mg/kg	gavage
POS	miltefosine (24 h before) + ascorbic acid	70 mg/kg + 60 mg/kg	gavage
Si30	ascorbic acid + miltefosine	30 mg/kg + 70 mg/kg	i.p. + gavage
Si60	ascorbic acid + miltefosine	60 mg/kg + 70 mg/kg	i.p. + gavage
Si120	ascorbic acid + miltefosine	120 mg/kg + 70 mg/kg	i.p. + gavage
Balb/c (repeated doses treatment)			
NC	distilled water	— (28 days)	gavage
IC	distilled water	— (28 days)	gavage
MT5.0	Impavido®	5.0 mg/kg (14 days)	gavage
MT2.5	Impavido®	2.5 mg/kg (28 days)	gavage
AA	ascorbic acid	15 mg/kg (28 days)	gavage
MT + AA	Impavido® + ascorbic acid	2.5 mg/kg + 15 mg/kg (28 days)	gavage

infected Balb/c mice received, simultaneously, 2.5 mg/kg miltefosine and 15 mg/kg ascorbic acid every day for 28 days; another group received only ascorbic acid in the same dose and in the same period of time. The dose of ascorbic acid was based on study of Kato et al. (2014). Negative control (distilled water) and infected control (animals with *L. infantum* infection) were also used. Treatment groups and doses are listed in Table 1.

2.5. Assays

2.5.1. Comet assay

It was collected from the tail of each animal approximately 5 µl peripheral blood and mixed with 100 µl low melting point agarose (0.5%). This mixture was then applied to pre-gelled slides with regular melting point agarose (1.5%). Then, coverslips were superimposed on these slides and refrigerated for 5 min. After solidification, slides were immersed in cold lysis solution (2.5M NaCl; 100 mM EDTA; 10 mM Tris (pH 10); 10% DMSO and 1% Triton X-100) and refrigerated for overnight at 4 °C. Slides were then incubated for 20 min in alkaline buffer (10M NaOH, 0.2M EDTA and distilled water, pH 13.0) followed by electrophoresis for 25 min at 25 V (0.72 V/cm)/300 mA. Slides were neutralized (0.4M Tris/HCl, pH 7.5), dried at room temperature and fixed with absolute ethanol for 4 min (Singh et al., 1988; Tice et al., 2000). Slides were stained with ethidium bromide (20 µg/ml) and analyzed by fluorescence microscopy (Olympus BX61).

For each animal, we analyzed 100 nucleoids, considering their size and quantity of DNA in the comet. DNA damage was classified in five levels: Class 0: no damage (< 5%); Class 1: low damage (5–20%); Class 2: medium damage (21–40%); Class 3: high damage (41–94%) e Class 4: total damage (> 95%). Damage scores were calculated by multiplying the number of nucleoids in each class by their respective class value, using the following equation:

$$\text{Score} = [(0 \times n0) + (1 \times n1) + (2 \times n2) + (3 \times n3) + (4 \times n4)] \,/\, \text{total cell number}$$

2.5.2. Micronucleus test

The femurs were removed for extraction of the bone marrow using 1 ml fetal bovine serum. Cell suspension was centrifuged for 5 min at 1000 rpm and the supernatant was discarded. Slide smearing (Schmid, 1975) was conducted with the remaining material. Slides were stained using hematological dye Panoptic kit (Laborclin, Pinhais, Paraná, Brasil) to differentiate polychromatic erythrocytes (PCE) from normochromatic erythrocytes (NCE).

To investigate micronucleus, 2000 polychromatic erythrocytes were analyzed for each animal in optical microscope magnification of 1000x (Carl Zeiss Primo Star). The ratio PCE/NCE (polychromatic erythrocytes and normochromatic erythrocytes) was calculated using 1000 erythrocytes for each animal to estimate toxicity for miltefosine and ascorbic acid.

Percentage of reduction in DNA damage induced by miltefosine, modulated by ascorbic acid, was calculated according to Waters et al. (1990) using the following formula: % Reduction = (CP – CT/CP - CN) x 100, where CP corresponds to the average damage of the group treated only with miltefosine, CT is the average damage observed in groups treated with ascorbic acid plus miltefosine and CN is the average damage observed in the negative control (distilled water).

2.5.3. Limiting dilution assay

Parasitic burden of infected animals was determined by the limiting dilution assay. Organs were collected and homogenized individually in 1.0 ml Schneider's Drosophila medium (Sigma-Aldrich, USA) supplemented with 10% inactivated fetal bovine serum (GIBCO, USA). The suspension with the homogenized organ was diluted 1:2 in the first well, which contained 100 µl of the medium, making up a total volume of 200 µl of suspension. From the first well, 100 µl of the suspension was withdrawn and the 1:2 dilution continued until the 12th well of the 96-well plate. This assay was conducted in duplicates. After 14 days, the sample from each well was analyzed and defined as positive or negative depending on the presence or absence of promastigotes in the well. The final titre was defined as the highest dilution for which the well contained at least one active parasite. The number of parasites per gram of the homogenate was calculated as follows: the reciprocal of the last positive titre by the total homogenized volume of the organ x dilution factor divided by the weight in grams of the homogenized organ. The viable parasitic burden was expressed as the number of leishmania per gram of homogenized organ (Rodrigues et al., 2009).

2.5.4. Superoxide dismutase activity

To analyze Superoxide Dismutase (SOD) activity, we collected blood from infected animals via cardiac puncture. Blood remained at rest for 30 min at 25 °C. It was then centrifuged at 2,000 g for 15 min at 4 °C and the serum was withdrawn. Subsequently, the Superoxide Dismutase Assay Kit (Chemical Cayman, MI, USA) was employed following the manufacturer's instructions.

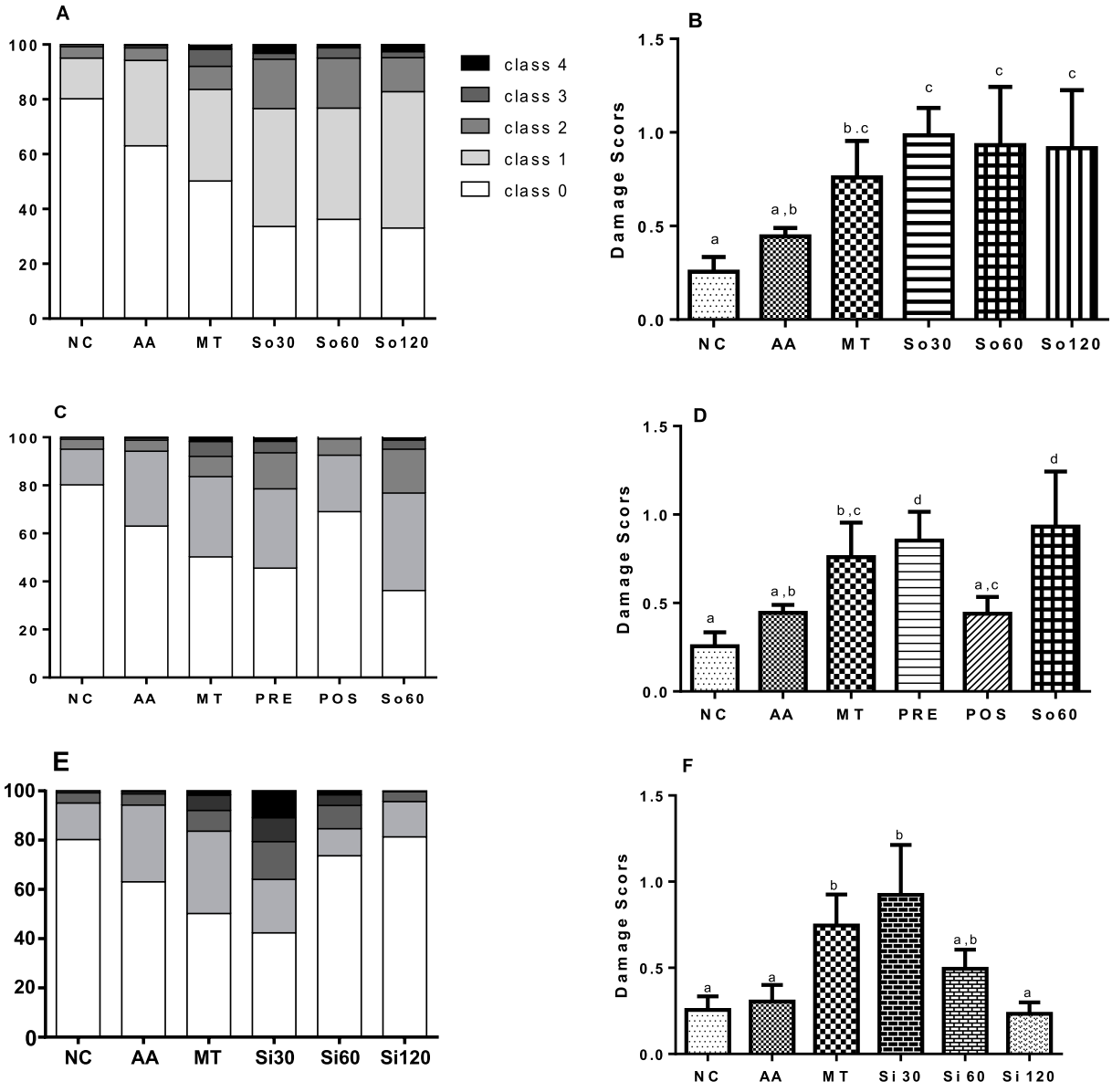

Fig. 1. Analysis of the antigenotoxic activity of ascorbic acid administered by gavage and intraperitoneally in Swiss mice on the genotoxic damages induced by the acute treatment with miltefosine (Chemical Cayman).

2.6. Statistical analysis

Data normality was assessed by Kolmogorov-Smirnov test. Numeric data obtained by comet assays, citotoxicity, parasitic burden and SOD Activity had a normal distribution and were submitted to one-way analysis of variance (ANOVA), followed by Tukey's *ad hoc* test. Micronucleus data were submitted to chi-square test (χ^2). All data were analyzed using GraphPad Prism 6.01 software, with values considered significant when $p < 0.05$.

3. Results

Through the comet assay, our results show that ascorbic acid did not have a protective effect over damage caused by miltefosine at the dose of 70 mg/kg (MT) when animals were treated via gavage and simultaneously (30, 60 and 120 mg/kg) (Fig. 1A and B). There was also no reduction in damage when ascorbic acid was administered 24 h prior to the MT group (PRE). However, the group treated with ascorbic acid 24 h after treatment with miltefosine (POS) showed reduction of damage near baseline (Fig. 1C and D).

The PCE/NCE ratio revealed that miltefosine and ascorbic acid were not cytotoxic at any of the doses tested ($p > 0.05$). When analyzing the antimutagenic activity of the ascorbic acid administered via gavage, none of the treatments (So30, So60, So120, PRE and POS) showed a protective effect of this antioxidant on DNA damage caused by miltefosine (Table 2).

When the same doses were tested intraperitoneally, we observed the protective effect of ascorbic acid over genotoxic damage caused by miltefosine, so that the higher dose (120 mg/kg) significantly reduced DNA damage at baseline, even though damage reduction could be observed from the intermediate dose (Fig. 1E and F).

The antimutagenic activity of ascorbic acid when administered intraperitoneally in mice was also analyzed. The same groups (Si60 and Si120) showed a significant decrease in micronuclei frequency, with 70.4% and 77.8% reduction, respectively, revealing the protective effect of ascorbic acid at these doses when administred intraperitoneally. The PCE/NCE ratio again demonstrated that miltefosine and ascorbic acid were not cytotoxic at any of the doses tested ($p > 0.05$), as shown in Table 3.

Based on these results, we sought to investigate whether this

Table 2
Analysis of the protective effect of orally administered ascorbic acid in Swiss mice on the mutagenic damage induced by acute treatment with miltefosine (Chemical Cayman).

GROUPS	PCEMNs	‰ MEDIA ± SD	PCE/NCE
NC	26	2.6 ± 0.42[a]	1.00
AA	25	2.5 ± 0.35[a]	1.16 [ns]
MT	53	5.3 ± 1.40[b]	0.87 [ns]
So30	50	5.0 ± 2.15[b]	1.14 [ns]
So60	37	3.7 ± 0.91[b]	1.32 [ns]
So120	40	4.4 ± 2.31[b]	0.73 [ns]
PRE	44	4.4 ± 0.65[b]	1.16 [ns]
POS	45	4.5 ± 0.79[b]	1.17 [ns]

Different letters denote statistical difference between the groups by χ^2 test.
[ns] absence of statistical difference when compared to the negative control group by ANOVA, followed by Tukey's *ad hoc* test.
NC: negative control (distilled water); **AA**: ascorbic acid (60 mg/kg); **MT**: miltefosine (70 mg/kg); **So30, So60** and **So120**: groups treated simultaneously with miltefosine (70 mg/kg) and ascorbic acid via gavage at the doses of 30 mg/kg, 60 mg/kg and 120 mg/kg, respectively; **PRE**: Group pretreated (24 h) with ascorbic acid (60 mg/kg) and later with miltefosine (70 mg/kg); and, **POS**: Group treated with miltefosine (70 mg/kg) and later (24 h) with ascorbic acid (60 mg/kg). To determine micronuclei frequency, 2000 polychromatic erythrocytes were counted per animal, and 1000 cells were counted for polychromatic and normochromatic erythrocytes ratio (PCE/NCE).

Table 3
Protective effect of ascorbic acid administered intraperitoneally in Swiss mice on the mutagenic damage induced by acute treatment with miltefosine (Chemical Cayman).

GROUPS	PCEMNs	‰ MEDIA ± SD	% DAMAGE REDUCTION	PCE/NCE
NC	26	2.6 ± 0.42[a]	–	1.00
AA	25	2.5 ± 0.35[a]	–	1.16 [ns]
MT	53	5.3 ± 1.40[b]	–	0.87 [ns]
Si30	49	4.9 ± 1.08[b]	–	1.30 [ns]
Si60	34	3.4 ± 0.96[a]	70.4	1.32 [ns]
Si120	32	3.2 ± 1.20[a]	77.8	1.28 [ns]

Different letters denote statistical difference between the groups by χ^2 test.
[ns] absence of statistical difference when compared to the negative control group by ANOVA, followed by Tukey's *ad hoc* test.
NC: negative control (distilled water); **AA**: ascorbic acid (60 mg/kg); **MT**: miltefosine (70 mg/kg); Simultaneous treatment with miltefosine (70 mg/kg) and ascorbic acid via i.p. at the doses of 30 mg/kg (**Si30**), 60 mg/kg (**Si60**) and 120 mg/kg (**Si120**). To determine micronuclei frequency, 2000 polychromatic erythrocytes were counted per animal, and 1000 cells were counted for polychromatic and normochromatic erythrocytes ratio (PCE/NCE).

protective effect of ascorbic acid also occurred in animals infected with *L. infantum*. Our results showed that in both doses tested (5.0 and 2.5 mg/kg/day), miltefosine (Impavido®) causes genomic lesions ($p < 0.01$). The genotoxic effect of miltefosine is observed both at the lowest dose with higher exposure (2.5 mg/kg for 28 days) and at twice the dose and half the treatment time (5.0 mg/kg for 14 days). It is also observed in Fig. 2 that animals receiving daily doses of miltefosine (2.5 mg/kg) and ascorbic acid (15 mg/kg) had a reduction in the damage score, reaching the basal levels of the negative control ($p > 0.05$). Table 4 shows that these same animals presented no increase in micronucleus frequency, revealing that at the tested doses miltefosine does not induce mutations. Likewise, neither *L. infantum* infection nor tested doses of miltefosine and ascorbic acid were able to alter the activity of the SOD enzyme ($p > 0.05$) (Fig. 3).

The action of miltefosine (Impavido®) on parasitic burden in animals infected with *L. infantum* is shown in Fig. 4, which shows that miltefosine (Impavido®) was effective in reducing the parasitic burden on the lymph node ($p < 0.0001$) by 86% and 88% at doses of 5 mg/kg and 2.5 mg/kg, respectively. When ascorbic acid was administered concurrently with antileishmanial miltefosine (Impavido®) (MT + AA), we found that there was no interference in drug efficacy, resulting in 81% reduction in parasite burden. Animals treated only with ascorbic acid (AA) also had a significant reduction in the parasitic burden of the lymph node (84%). In the spleen, only the 5.0 mg/kg dose (MT5.0) was effective in reducing parasitic burden, resulting in 99% *Leishmania* reduction ($p < 0.05$).

4. Discussion

Many studies have investigated ascorbic acid's potential in modulating the harming effects of free radicals through its antioxidant activity, helping to prevent or delay the development of certain cancers (da Mata et al., 2016), cardiovascular diseases (Zhang et al., 2014) and other diseases related to oxidative stress (Harrison, 2012), in addition to aging (Monacelli et al., 2017).

Taking into consideration that our previous study demonstrated that miltefosine can induce oxidation in the nitrogenous bases of DNA (Castelo Branco et al., 2016), this work evaluated the ability of ascorbic acid to reduce the genetic damage caused by this antileishmanial. For this, the erythropoietic cytotoxicity of miltefosine and ascorbic acid was initially evaluated, in which no change in PCE/NCE ratio was observed in any of the treatment groups, which shows that the doses studied were not cytotoxic.

Firstly, the antioxidant effect of ascorbic acid was evaluated for both vias, gavage and i.p. routes, when administered simultaneously with miltefosine in uninfected animals. The results revealed that ascorbic acid does not present antigenotoxic properties for any of the doses tested when given via gavage. On the other hand, through via i.p., highest doses (60 and 120 mg/kg) presented a protective effect over DNA, corroborating with other studies that reported the antigenotoxic effect of ascorbic acid when administered intraperitoneally (Devi and Latha, 2011; García-Rodríguez et al., 2016; de Jesus et al., 2018). These findings are justified by the fact that intravenous administration of ascorbic acid produces plasma concentrations of 30–70 times higher than gavage doses (Padayatty et al., 2004; Chen et al., 2007; Kato et al., 2014).

Besides simultaneous treatment, we also analyzed two other forms of animal treatment with ascorbic acid: pre and post treatment. In the pre treatment group, no protective effect of ascorbic acid was observed, which may be related to the low plasma concentration added to the short half-life of this antioxidant, which is of approximately 10 h (Schwedhelm et al., 2003). Studies demonstrating the antimutagenic capacity of ascorbic acid in pre treatment have administered the antioxidant more than once before treatment and simultaneously with the drug (Giri et al., 1998; Vijayalaxmi and Venu, 1999; Cheng et al., 2003; Franke et al., 2005; Roy et al., 2008). On the other hand, in the post treatment group there was a reduction in genotoxic damage (Fig. 1D), which approached baseline control, with an increase in class 0 score (Fig. 1C). However, in this group, the frequency of micronucleated cells was not reduced, which may be related to the low serum level of ascorbic acid in this exposure route.

Based on the results obtained in uninfected animals, the ascorbic acid response was investigated in animals infected with *L. infantum* under repeated doses treatment with miltefosine (Impavido®). We observed that, although there was no significant difference between the damage scores (Fig. 2), ascorbic acid (15 mg/kg/day) showed a slight reduction in the damage caused by the drug (2.5 mg/kg/day). In addition, corroborating with single dose treatment data, in the repeated doses treatment the miltefosine (Impavido®) was genotoxic, although it was not mutagenic at the doses studied (5.0 and 2.5 mg/kg/day) (Table 4). Both damage (genotoxic and mutagenic) induced by miltefosine (Impavido®) were demonstrated by our group in single dose tretament. We have suggested that miltefosine (Impavido®) is a drug that damages DNA by oxidative stress, with guanine oxidation being the most common damage, forming 8-oxo-7,8-dihydroguanine (Castelo

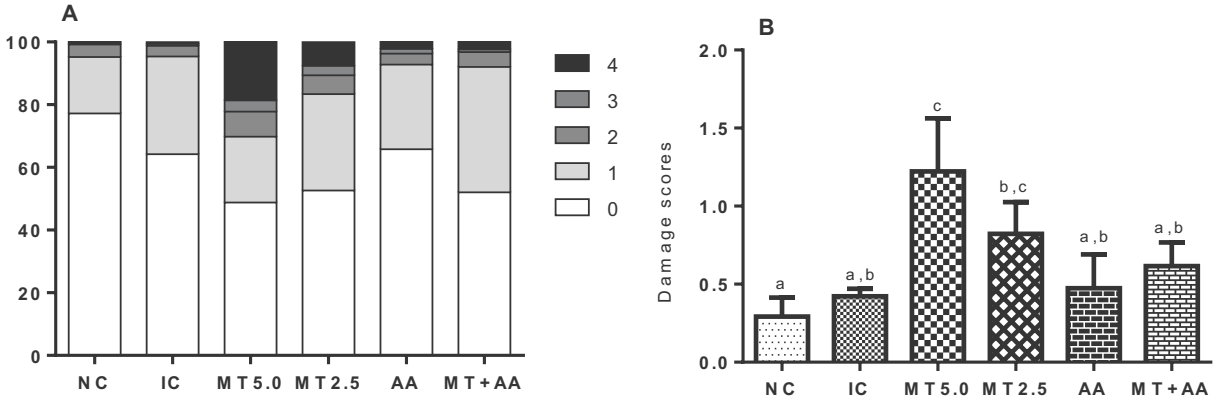

Fig. 2. Analysis of the antigenotoxic activity of ascorbic acid in Balb/c mice infected with *L. infantum* on the genotoxic damages induced by the cronic treatment with miltefosine (Impavido®).
Different letters denote statistical difference between the groups by ANOVA, followed by Tukey's *ad hoc* test.
A: Frequency of nucleoid classes observed by the Comet Assay; **B:** and their respective damage scores.
NC: negative control (distilled water); **IC**: infected control; **MT5.0**: treatment with miltefosine (5.0 mg/kg for 14 days); **MT2.5:** treatment with miltefosine (2.5 mg/kg for 28 days); **AA**: treatment with ascorbic acid (15 mg/kg for 28 days); **MT + AA**: treatment with ascorbic acid + miltefosine (15 mg/kg + 2.5 mg/kg for 28 days, respectively).

Table 4
Percentage of micronucleated cells in the bone marrow of Balb/c mice, infected with L. infantum, in chronic treatment with orally introduced miltefosine (Impavido®) and antioxidant effect of ascorbic acid.

GROUPS	PCEMNs	‰ MEDIA ± SD	PCE/NCE
NC	27	2.7 ± 0.89[a]	0.99
IC	31	3.1 ± 1.92[a]	1.14 [ns]
MT5.0	35	4.4 ± 1.50[a]	1.05 [ns]
MT2.5	28	2.8 ± 2.30[a]	1.27 [ns]
AA	28	3.5 ± 2.16[a]	1.06 [ns]
MT + AA	22	2.8 ± 2.08[a]	1.09 [ns]

[a] Absence of statistical difference between the groups by χ^2 test.
[ns] absence of statistical difference ($p > 0.05$) compared to the negative control group by ANOVA, followed by Tukey's *ad hoc* test.
NC: negative control (distilled water); **IC**: infected control; **MT5.0:** treatment with miltefosine (5.0 mg/kg for 14 days); **MT2.5**: treatment with miltefosine (2.5 mg/kg for 28 days); **AA**: treatment with ascorbic acid (15 mg/kg for 28 days); **MT + AA**: treatment with miltefosine + ascorbic acid (2.5 mg/kg + 15 mg/kg for 28 days, respectively). To determine micronuclei frequency, 2000 polychromatic erythrocytes were counted per animal, and 1000 cells were counted for polychromatic and normochromatic erythrocytes ratio (PCE/NCE).

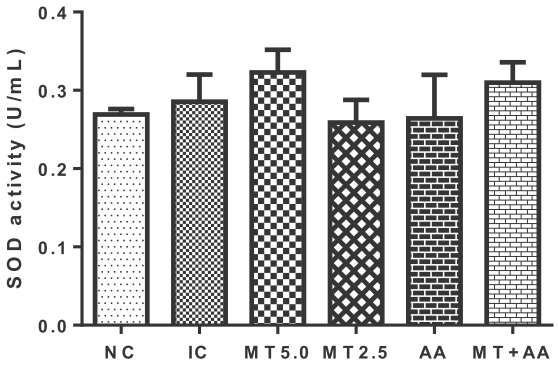

Fig. 3. Activity of antioxidant enzyme Superoxide dismutase (SOD).

Branco et al., 2016).

In response to this stress and to maintain genomic stability, cells activate DNA repair processes and transcription factors, which in turn modulate ROS-scavenging expression levels. Among them, the 8-oxoguanine glycosylase (OGG1), muty DNA glycosylase (MUTYH) and homologous mutT 1 (MUTH1) enzymes, which constitute the 8-oxoG repair pathway (Barnes and Lindahl, 2004), are activated, as well as the transcription factor Yap1, which positively regulates several genes related to the antioxidant defense (catalase, superoxide dismutase and glutathione peroxidase) (Rowe et al., 2008). Thus, our data suggest that genomic damage induced by miltefosine (Impavido®) may have been efficiently corrected by the DNA repair cell system, probably because the doses in the animals were lower than the equivalent therapeutic doses used in humans, as reported by Nair and Jacob (2016). In addition, we did not observe an increase in the activity of the SOD enzyme (Fig. 3), supporting the lack of mutagenic effect of this antileishmanial, including in the group with the infection alone (IC). Similar results were obtained by Moreira et al. (2017) who also found that the infection alone does not affect the levels of this enzyme.

Considering that some antileishmanial drugs exert their activity through the induction of oxidative stress (Basu et al., 2006; Castelo Branco et al., 2016; Moreira et al., 2017), we also evaluated whether the antioxidant action of ascorbic acid interferes with the antileishmanial activity of miltefosine. Our results have shown that the concurrent administration of ascorbic acid and miltefosine (Impavido®) did not interfere in the drug efficacy, as we have verified a reduction of 81% in the lymph node parasitic burden (Fig. 4). Similar results were observed in mice treated with antileishmanial Glucantime® and treated with the same dose of ascorbic acid used in this study (Kato et al., 2014); furthermore, the authors still observed that the antioxidant improved the drug's efficacy in suppressing parasites. It is also interesting to highlight that in the present study we also observed that infected animals treated only with ascorbic acid also had a significant reduction of *L. infantum* in the lymph node (84% reduction), showing that this antioxidant contributes to the fight against infection (Fig. 4).

In infected animals treated only with the drug, we found that, even at a dose lower than the equivalent human dose, miltefosine (Impavido®) was effective in reducing the parasite burden of *L. infantum*, with a reduction of 86% (at a dose of 5.0 mg/kg) and 88% in the lymph node (2.5 mg/kg) and 99% in the spleen (5.0 mg/kg) (Fig. 4). Other studies have also shown the efficacy of miltefosine action on the parasite burden in the spleen of *L. infantum* infected animals, with a reduction of 70 and 75% (10 and 20 mg/kg respectively for 5 days) (Kuhlencord et al., 1992), 78% (20 mg/kg for 5 days) (Le Fichoux et al., 1998) and 86% (20 mg/kg for 10 days) (Reimão et al., 2015). Therefore, our data show that lower doses of miltefosine during 14 days of treatment have equal or better efficacy in reducing parasitic burden.

In summary, our data showed that ascorbic acid was able to reduce the parasite load, besides protecting the mammalian DNA against

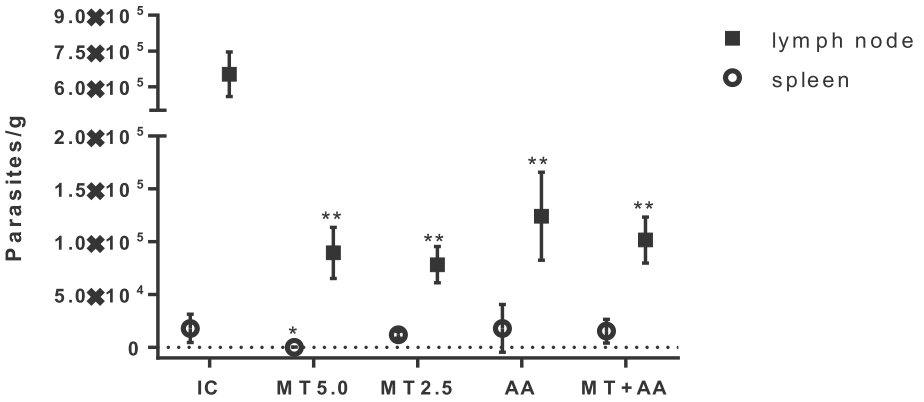

Fig. 4. Effect of ascorbic acid on the parasite load of Balb/c mice infected with L. infantum and treated with miltefosine (Impavido®) and ascorbic acid.

genotoxic damage caused by the antileishmanial, opening perspectives for future studies in order to minimize the harmful effects on DNA, besides being potentially capable of reducing infection.

5. Conclusion

Our data show that ascorbic acid reduces the genetic damage induced by miltefosine, without decreasing its antileishmanial activity. In addition, the results reveal that miltefosine is effective in reducing the parasitic burden of *L. infantum* at lower doses than others previously reported.

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