Genistein and Ascorbic Acid Reduce Oxidative Stress-Derived DNA Damage Induced by the Antileishmanial Meglumine Antimoniate

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ABSTRACT Meglumine antimoniate (Glucantime) is a pentavalent antimonial used to treat leishmaniasis, despite its acknowledged toxic effects, such as its ability to cause oxidative damage to lipids and proteins. Recently, our group demonstrated that meglumine antimoniate causes oxidative stress-derived DNA damage. Knowing that antioxidants modulate reactive oxygen species, we evaluated the capacity of genistein and ascorbic acid for preventing genotoxicity caused by meglumine antimoniate. For that, mice ($n = 5/\text{group}$) received genistein (via gavage) in doses of 5, 10, and 20 mg/kg for three consecutive days. After this period, they were treated with 810 mg/kg meglumine antimoniate via intraperitoneal (i.p.) route. Furthermore, mice ($n = 5/\text{group}$) simultaneously received ascorbic acid (i.p.) in doses of 30, 60, and 120 mg/kg and 810 mg/kg meglumine antimoniate. We also conducted post- and pretreatment assays, in which animals received ascorbic acid (60 mg/kg) 24 h prior to or after receiving meglumine antimoniate. Genomic instability and mutagenicity were analyzed through conventional comet assay and enzymatic assay using formamide pyrimidine DNA glycosylase (Fpg) enzyme, as well as the micronucleus test, respectively. Meglumine antimoniate induced an increase in the DNA damage after digestion with Fpg, reinforcing its mutagenic potential by oxidizing DNA bases, which was prevented by genistein. Similarly, ascorbic acid was capable of reducing mutagenic effects in simultaneous treatment as well as in posttreatment. Therefore, our results demonstrate that both compounds are efficient in preventing mutations in mammalian cells treated with meglumine antimoniate.

KEYWORDS antioxidants, antimutagenicity, soy isoflavone, vitamin C, pentavalent antimonial

Leishmaniasis are chronic and noncontagious infectious diseases that are broadly distributed around the world and caused by more than 20 species of protozoans from the genus *Leishmania* (1). Clinical manifestations vary according to the parasite species causing the disease, with three main forms: (i) cutaneous leishmaniasis (CL), the most common form, which causes ulcers in the skin; (ii) mucocutaneous leishmaniasis (ML), which affects skin and mucous membranes, causing total or partial damage to affected areas; and (iii) visceral leishmaniasis (VL), also known as kala-azar, which is the most serious clinical form and affects internal organs, sometimes being fatal if not adequately treated (1, 2). Estimates of about 50,000 to 90,000 new cases of VL throughout the world are predicted every year. In 2015, over 90% of all cases were recorded in Brazil, Ethiopia, India, Kenya, Somalia, Sudan, and South Sudan. Regarding CL, about 0.6 to 1 million new cases occur yearly, of which 2/3 are from six countries located in South America, the Mediterranean bay, the Middle East, and Central Asia (1).

Despite its high incidence and alarming clinical consequences, leishmaniasis is still

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a neglected disease. Meglumine antimoniate (Glucantime) is one of the drugs most used to treat leishmaniasis by recommendation of the World Health Organization (3–5), even though its mechanisms of action are still unclear and its toxic effects are well known (6, 7). Among the main hypotheses on the mechanism of action of meglumine antimoniate, one suggests that Sb^V acts as a prodrug, which is metabolized and reduced to its more toxic trivalent form (Sb^{III}). In vertebrates, this reduction can be mediated by thiols, such as glutathione (GSH) and cysteine or cysteine-glycine, presented in the cytosol and phagolysosomes of the macrophages, respectively. On the other hand, the reduction can occur within the parasite through the trypanothione- and thiol-dependent reductase enzyme (8–10). Hence, the antimonial induces the production of reactive oxygen species (ROS) and, consequently, oxidative stress (10–14). This leads to disturbances in the normal redox state of the cell, damaging the main biomolecules such as proteins, lipids (15), and DNA (16).

To control generation of ROS, cells possess an elaborate enzymatic system involving many enzymes with antioxidant activities, such as glutathione peroxidase (GPx), superoxide dismutase (SOD), glutathione S-transferase (GST), and catalase (CAT), whose function is to stop buildup of ROS in the organism and prevent these agents from causing damage to biomolecules (17, 18). Some nonenzymatic compounds also show antioxidant activity and are obtained mainly through diet, such as α -tocopherol (vitamin E), ascorbic acid (vitamin C), carotenoids, and flavonoids, as well as peptides with thiol groups, such as GSH, among others (19, 20). Interestingly, studies have demonstrated that antioxidant compounds such as ascorbic acid and genistein can also reduce mutations induced by different mutagens (21, 22).

Considering that our research group has demonstrated that meglumine antimoniate causes oxidation of nitrogenous bases on DNA and alters oxidant enzymes activities in BALB/c mice infected by *Leishmania* (*Leishmania*) *infantum*, leading to genomic instability and mutation fixation (23), and keeping in mind that antioxidant compounds neutralize deleterious actions of ROS on biomolecules (24, 25), the aim of this study was to evaluate the potential of two acknowledged antioxidants (genistein and ascorbic acid) in modulating genetic damage caused by the antileishmanial meglumine antimoniate.

RESULTS

Meglumine antimoniate induces oxidative stress-derived DNA damage. Figure 1 shows the frequencies of nucleoid classes observed by the conventional comet assay (Fig. 1A) and by comet assay followed by digestion using Fpg enzyme (Fig. 1B) in peripheral blood leukocytes from healthy mice treated with meglumine antimoniate. The frequency of the highest nucleoid classes (3 and 4) increased when comparing the group treated using meglumine antimoniate to the negative control (NC) ($P < 0.05$). There also was no significant difference between the positive control (PC) and the group treated with the antimonial, reiterating the drug's genotoxic potential (Fig. 1C). On the other hand, the comet assay followed by digestion with Fpg enzyme showed an increase in frequency for classes 2, 3, and 4 (Fig. 1B) compared to that found by conventional comet assay. This novel damage class distribution is reflected in DNA damage scores (Fig. 1C), in which the level of damage increased significantly after digestion with the endonuclease Fpg ($P < 0.001$), thus reinforcing that this antileishmanial causes damage to DNA by oxidation of bases.

Genistein and ascorbic acid reduce genotoxic damage caused by meglumine antimoniate. The comet assay in peripheral blood leukocytes of mice treated with genistein and meglumine antimoniate revealed an increase in the frequency of nucleoids 2, 3, and 4 in the group treated with meglumine antimoniate in relation to the vehicle control (VC) group ($P < 0.05$) (Fig. 2A). On the other hand, there is a significant difference ($P < 0.05$) between damage scores from the group receiving meglumine antimoniate (GLU group) (1.53 ± 0.30) and those obtained from groups pretreated with genistein at 5 mg/kg (1.11 ± 0.08), 10 mg/kg (0.9 ± 0.11), and 20 mg/kg (0.98 ± 0.06) (Fig. 2B). These results reveal the ability of genistein to reduce genomic instability

FIG 1 Frequencies of nucleoid classes observed in peripheral blood leukocytes from uninfected Swiss mice treated with meglumine antimoniate ($n = 5$ per group) by conventional comet assay (A) and comet assay followed by Fpg digestion (B) and their respective DNA damage scores (mean $\pm$ standard deviation [SD]) (C). Statistical differences were calculated using the Student t test (*, $P < 0.05$). NC, negative control (distilled water); PC, positive control (50 mg/kg cyclophosphamide); GLU, meglumine antimoniate (810 mg/kg).

induced by the antileishmanial, which showed a reduction of DNA damage of 51%, 78%, and 68%, respectively.

Regarding ascorbic acid, our data show an increase of class zero nucleoids in the groups treated simultaneously (S1, S2, and S3) (Fig. 3A) compared to the group treated with meglumine antimoniate ($P < 0.05$). Hence, damage scores from groups treated simultaneously with meglumine antimoniate and ascorbic acid (0.36 ± 0.09 , 0.27 ± 0.11 , and 0.39 ± 0.12) were significantly lower than those from the GLU group (0.70 ± 0.03) ($P < 0.01$), reaching a basal damage level similar to that for the negative control (0.25 ± 0.07) (Fig. 3B). Considering the percentage of DNA damage reduction (76%,

FIG 2 Frequency of nucleoid classes observed by comet assay in peripheral blood leukocytes of Swiss mice treated using genistein and meglumine antimoniate (A) and their respective damage scores (mean $\pm$ SD) (B) ($n = 5$ per group). Different letters (a, b, and c) indicate statistically significant differences ($P < 0.05$) by ANOVA followed by Tukey's *ad hoc* test. VC, vehicle control (1% DMSO); PC, positive control (50 mg/kg cyclophosphamide); GLU, meglumine antimoniate (810 mg/kg); G1, 5 mg/kg genistein plus 810 mg/kg meglumine antimoniate; G2, 10 mg/kg genistein plus 810 mg/kg meglumine antimoniate; G3, 20 mg/kg genistein plus 810 mg/kg meglumine antimoniate).

FIG 3 Frequencies of nucleoid classes and their respective DNA damage scores (mean $\pm$ SD) obtained by comet assay in peripheral blood leukocytes in Swiss mice treated with ascorbic acid and meglumine antimoniate. (A and B) Simultaneous treatment. S1, 30 mg/kg; S2, 60 mg/kg; S3, 120 mg/kg ascorbic acid plus 810 mg/kg meglumine antimoniate ($n = 5$ animals per group). (C and D) Pretreatment (60 mg/kg ascorbic acid 24 h prior to meglumine antimoniate [810 mg/kg]) and posttreatment (60 mg/kg ascorbic acid 24 h after meglumine antimoniate [810 mg/kg]) ($n = 5$ animals per group). Different letters (a, b, and c) indicate statistical difference ($P < 0.05$) by ANOVA followed by Tukey's *ad hoc* test.

95%, and 70%, respectively), we evaluated the effects of the intermediate dose (60 mg/kg) in simultaneous treatment, pretreatment, and posttreatment. The distribution of classes and respective damage scores are shown in Fig. 3C and D. Scores obtained from simultaneous treatment (0.27 ± 0.11) and posttreatment (0.22 ± 0.14) are significantly lower than those from the group treated with meglumine antimoniate (0.70 ± 0.03) and do not differ between each other or from those for the negative control. The procedures are thus equally efficient in reducing genomic damage caused by the antileishmanial. On the other hand, ascorbic acid administration prior to meglumine antimoniate did not present antigenotoxic action (score equal to 0.60 ± 0.16).

Genistein and ascorbic acid decrease the frequency of micronucleated cells.

Meglumine antimoniate did not have a cytotoxic effect on erythrocytes since the ratio of polychromatic erythrocytes (PCE) to normochromatic erythrocytes (NCE) was similar to that for the control. The frequencies of micronuclei in the positive control (50 mg/kg) and in the meglumine antimoniate group (810 mg/kg) also confirmed the mutagenic effect of these compounds. On the other hand, the three genistein doses were capable of significantly decreasing the antileishmanial-induced frequency of micronucleated cells ($P < 0.001$), reaching the basal levels in the vehicle control (Table 1). Tables 2 and 3 show the data from animals treated with ascorbic acid and meglumine antimoniate in simultaneous treatment and under pre- and posttreatment conditions. For the groups treated simultaneously, the three ascorbic acid doses showed efficiency in reducing meglumine antimoniate-induced micronuclei (Table 2). Notably, the intermediate dosage was able to decrease the frequency of micronuclei and also showed the highest potential to reduce DNA damage. Based on our results from simultaneous treatment, the protective effect of ascorbic acid was also evaluated under pre- and posttreatment conditions with the intermediate dose. We observed a decrease in the frequency of micronucleated cells in posttreatment, which did not show any significant difference from the simultaneous treatment. On the other hand, pretreatment with

TABLE 1 Frequency of micronucleated polychromatic erythrocytes and PCE/NCE ratio in the bone marrow of Swiss mice treated with genistein and meglumine antimoniate and of their respective controls

Group ^a	No.	MnPCE, % (mean $\pm$ SD) ^b	PCE/NCE ratio ^c
VC	33	3.3 $\pm$ 0.50 a	0.99
PC	306	30.6 $\pm$ 4.04 b	0.93 (ns)
GLU	144	14.4 $\pm$ 1.43 c	0.96 (ns)
G1	60	6.0 $\pm$ 0.12 a	0.99 (ns)
G2	42	4.2 $\pm$ 0.07 a	1.00 (ns)
G3	33	3.3 $\pm$ 0.05 a	0.99 (ns)

^a*n* = 5 animals/group; 2,000 erythrocytes per animal. VC, vehicle control (1%); PC, positive control (50 mg/kg cyclophosphamide); GLU, meglumine antimoniate (810 mg/kg); G1, genistein (5 mg/kg) plus meglumine antimoniate (810 mg/kg); G2, genistein (10 mg/kg) plus meglumine antimoniate (810 mg/kg); G3, genistein (20 mg/kg) plus meglumine antimoniate (810 mg/kg).

^bMnPCE, micronucleated polychromatic erythrocytes. Different letters indicate a statistically significant difference (*P* < 0.05) by ANOVA followed by Tukey's *ad hoc* test; *F* = 164.9.

^cns, no statistically significant difference compared to VC.

ascorbic acid was not able to reduce the mutagenic effect of the antileishmanial (Table 3).

DISCUSSION

Many studies have proposed that meglumine antimoniate acts as a prodrug. This model suggests that the pentavalent antimonial (Sb^V) is converted into its more toxic trivalent form (Sb^{III}) by thiols (8, 9). Evidence indicates that Sb^{III} compromises the redox potential of the cell, leading to ROS production and, consequently, increasing the oxidative stress (10–12, 15). Hence, the reduction of Sb^{III} has been mentioned as one possible mechanism for the drug's toxicity and for its therapeutic activity. This hypothesis is corroborated by our previous study, in which it was demonstrated that meglumine antimoniate induces mutations *in vivo* but not *in vitro* (16). These data also support a previous study that related genetic risk and oxidative stress in workers exposed to antimony trioxide (26).

The association between genetic alterations and oxidative damage was reinforced by a study by Cantanhêde and collaborators (27), who demonstrated that soy isoflavones are effective in reducing genomic lesions as well as the frequency of micronucleated cells induced by meglumine antimoniate. More recently, this idea was supported by demonstrating that meglumine antimoniate causes genetic instability by oxidation of nitrogenous bases in infected BALB/c mice treated for 20 days (20 mg/kg/day) (23). The score of damage caused by the antileishmanial increased in the comet assay that uses Fpg repair endonucleases. This assay allows recognition and removal of products generated from the oxidation of purine bases, therefore increasing

TABLE 2 Frequency of micronucleated polychromatic erythrocytes and PCE/NCE ratio in the bone marrow of Swiss mice treated simultaneously with ascorbic acid and meglumine antimoniate and of their respective controls

Group ^a	No.	MnPCE, % (mean $\pm$ SD) ^b	PCE/NCE ratio ^c
NC	33	3.5 $\pm$ 0.50 a	1.00
PC	306	30.6 $\pm$ 4.04 b	0.93 (ns)
GLU	144	14.4 $\pm$ 1.43 c	0.96 (ns)
S1	76	7.6 $\pm$ 1.29 d	0.98 (ns)
S2	64	6.4 $\pm$ 1.19 a, d	0.93 (ns)
S3	103	10.3 $\pm$ 1.48 d	0.87 (ns)

^a*n* = 5 animals/group; 2,000 erythrocytes per animal. NC, negative control (distilled water); PC, positive control (50 mg/kg cyclophosphamide); GLU, meglumine antimoniate (810 mg/kg); S1, ascorbic acid (30 mg/kg) plus meglumine antimoniate (810 mg/kg); S2, ascorbic acid (60 mg/kg) plus meglumine antimoniate (810 mg/kg); S3, ascorbic acid (120 mg/kg) plus meglumine antimoniate (810 mg/kg).

^bMnPCE, micronucleated polychromatic erythrocytes. Different letters indicate a statistically significant difference (*P* < 0.05) by ANOVA followed by Tukey's *ad hoc* test; *F* = 120.4.

^cns, no statistically significant difference compared to NC.

TABLE 3 Frequency of micronucleated polychromatic erythrocytes and PCE/NCE ratio in the bone marrow of Swiss mice treated with ascorbic acid and meglumine antimoniate under simultaneous treatment, pretreatment, and posttreatment conditions, with their respective controls

Group ^a	No.	MnPCE, % (mean $\pm$ SD) ^b	PCE/NCE ratio ^c
NC	35	3.5 $\pm$ 0.50 a	1.00
PC	306	30.6 $\pm$ 4.04 b	0.93 (ns)
GLU	144	14.4 $\pm$ 1.43 c	0.96 (ns)
S2	64	6.4 $\pm$ 1.19 a, d	0.93 (ns)
PRE	110	11.0 $\pm$ 1.00 c, e	0.93 (ns)
POS	81	8.1 $\pm$ 0.96 d, e	0.95 (ns)

^a*n* = 5 animals/group; 2,000 erythrocytes per animal. NC, negative control (distilled water); PC, positive control (50 mg/kg cyclophosphamide); GLU, meglumine antimoniate (810 mg/kg); S2, ascorbic acid (60 mg/kg) plus meglumine antimoniate (810 mg/kg); PRE, ascorbic acid (60 mg/kg) plus meglumine antimoniate (810 mg/kg); POS, meglumine antimoniate (810 mg/kg) plus ascorbic acid (60 mg/kg).

^bMnPCE, micronucleated polychromatic erythrocytes. Different letters indicate a statistically significant difference (*P* < 0.05) by ANOVA followed by Tukey's *ad hoc* test; *F* = 129.2.

^cns, no statistically significant difference compared to NC.

DNA fragmentation and, consequently, the damage score detected by the conventional comet assay (28).

This present study focused on the capacity of recognized antioxidants to modulate DNA damage induced by meglumine antimoniate. First, our data show that pentavalent antimony causes damage to the DNA of mice treated intraperitoneally with a high dose and for a short period of time. These results support that the reduction of pentavalent antimony occurs, at least in part, in phagolysosomes of macrophages (8), since our experimental model allows a greater and faster exposure of the peritoneal macrophages. Furthermore, some studies found large amounts of residual Sb^{III} in pentavalent antimony, up to 15 to 30% (14, 29, 30). Hence, considering that animals received nearly 195 mg/kg of Sb^{III}, the genotoxicity observed in the present study could also be due to the high level of residual trivalent antimony in the commercial meglumine antimoniate.

Second, considering that the mutagenic effects of meglumine antimoniate are mediated by oxidative processes, here we investigated the capacity of two antioxidants, genistein and ascorbic acid, to reduce/prevent antileishmanial meglumine antimoniate-induced genetic damage. The first one, genistein, is a soybean-derived isoflavonoid that is considered an important agent in cancer prevention (31, 32); this effect is associated with its recognized antioxidant capacity (33–36).

Our data demonstrated that pretreatment with doses of 5 mg/kg, 10 mg/kg, and 20 mg/kg genistein was capable of decreasing meglumine antimoniate-induced genotoxicity, revealing that the pretreatment with genistein was able to modulate the reactive oxygen species generated by the antileishmanial meglumine antimoniate. We suggest that the genistein antimutagenic activity is correlated with its capacity to capture free radicals generated after metabolic activation from the pentavalent antimonial form to its trivalent form, thus protecting genetic material from oxidative damage. Corroborating this idea, Pugalendhi et al. (22) verified that a dose of 20 mg/kg genistein was able to protect from mutagenic effects induced by 7,12-dimethylbenzo[*a*]anthracene. Furthermore, Ali et al. (37) verified that genistein administration for 21 days reduced lipid peroxidation, protein carbonylation, and DNA damage induced by *N*-nitrosodiethylamine (0.1 mg/ml) in rat hepatocytes.

According to Ding and Liu (38), genistein acts by increasing the expression of genes involved in the detoxification of ROS, such as superoxide dismutase, glutathione peroxidase, and catalase. This hypothesis is supported by Suzuki et al. (39), who observed that genistein was able to significantly increase expression of the gene *GPx-1* in human prostate cancer cell lines (LNCaP and PC-3), contributing to antioxidant defense and inhibition of proliferation of these cells. Gong et al. (40) also verified an increase in the activity of these enzymes by genistein against cadmium-induced neurotoxic damage in Wistar mice, thus demonstrating the protective potential of this isoflavonoid and its capacity to maintain redox equilibrium.

Another possible mechanism to explain the antimutagenic effect of genistein is related to its ability to stimulate expression of genes involved in DNA repair, such as *BCRA1* (41) and *ATM* and *p53* (42). This mechanism was also reported by Song et al. (43), who studied the effect of genistein in preventing and repairing radiation-induced DNA damage in HL-7702 cells. The authors noted that genistein was able to increase expression of genes involved in DNA repair, such as *hHR23A*, *HUS1*, *RAD1*, and *RAD9*. Therefore, the data here presented support the hypothesis that the antileishmanial causes DNA damage by oxidation of its nitrogenous bases, which was reduced by genistein through its antioxidant activity.

Our data also revealed that ascorbic acid was able to reduce meglumine antimoniate-induced genomic damage, corroborating previous studies that observed a protective effect of ascorbic acid against mutagenic damage induced by other stressors such as cyclophosphamide (44), arsenic trioxide (45), 3,5-dimethylaminophenol (46), and vanadium pentoxide (47). Ascorbic acid is a nonenzymatic antioxidant obtained from the diet and is essential to humans due to its participation in several physiological activities (48). This vitamin promotes beneficial effects on health, since its antioxidant activity prevents development of certain types of cancer, cardiovascular diseases, and other illnesses related to oxidative stress (49–51). According to Vijayalaxmi and Venu (21), as well as Farghaly and Abo-Zeid (52), the antigenotoxic and antimutagenic abilities are due to its capacity to convert reactive compounds to less toxic and more easily excreted products, as well as to capture free radicals before they interact with other molecules, protecting them from oxidative damage.

Our data also agree with those of Kato et al. (14), who demonstrated that ascorbic acid promotes hepatoprotection in animals treated with meglumine antimoniate due to its capacity to decrease the peroxidase enzyme activity. In addition, these authors showed that cotreatment with ascorbic acid and meglumine antimoniate did not interfere with the antileishmanial activity of the drug in mice infected by *Leishmania infantum*, suggesting that this antioxidant may be part of a therapeutic strategy effective against toxicity caused by meglumine antimoniate. Here, our data emphasize that this protection includes DNA protection against oxidative stress.

Besides its well-known ability to eliminate ROS (21, 46, 47), other studies suggest that ascorbic acid can act as a cofactor of transcription factors, upregulating genes involved in DNA repair (53–55). These authors demonstrated that ascorbic acid is able to increase expression of *SOD3* (encoding superoxide dismutase 3) and *OGG1* (encoding 8-oxoguanine DNA glycosylase) by activation of NRF2 (factor 2 nuclear transcription factor related to erythroid 2). Moreover, Alevva et al. (56) have verified that dietetic ingestion of ascorbic acid (600 mg/day) in cells from volunteers treated with hyperbaric oxygen (HBO) induced the expression of important genes for cell detoxification, such as *GSTZ1* (encoding glutathione *S*-transferase zeta 1), genes encoding multifunctional enzymes important to detoxification of electrophilic molecules, and *NUDT1* (encoding nucleoside diphosphate linked moiety X motif 1), a gene encoding 7,8-di-hydro-8-oxoguanine triphosphatase, which hydrolyzes oxidized purines. Additionally, Cooke et al. (57) observed a significant decrease of 8-oxo-G in DNA of cells from volunteers supplemented with ascorbic acid (500 mg/day), thus highlighting that ascorbic acid probably stimulates repairs to oxidized bases in DNA due to its redox properties.

Briefly, based on our data regarding antigenotoxic and antimutagenic effects detected in simultaneous treatment, we suggest that ascorbic acid, a strong reducing agent, acts by eliminating reactive oxygen species generated during conversion of pentavalent antimonial to its trivalent form, thus protecting genetic material from oxidative damage. Additionally, ascorbic acid administered 24 h after the antileishmanial was able to reduce DNA damage, suggesting that this antioxidant also acts by repairing damage, possibly by inducing expression of genes related to the DNA repair system, halting mutation fixation. Therefore, the protective effect of ascorbic acid against oxidative stress-derived DNA damage caused by meglumine antimoniate may be due to both mechanisms. Future studies must be performed to evaluate the

differential expression of genes involved in both molecular responses to oxidative DNA damage caused by meglumine antimoniate.

We have also shown that ascorbic acid (60 mg/kg) is not able to decrease the deleterious effects on genomic instability when administered 24 h prior to the mutagenic drug. This probably occurs due to ascorbic acid's half-life; it is eliminated from an organism 10 to 12 h after consumption (58, 59). In addition, we emphasize that the antigenotoxic effects of genistein and ascorbic acid were observed at all doses, but there was no dose-response effect. Many different factors can be related to this fact, such as the pathological and physiological conditions of the organism, pharmacokinetics and bioavailability of the drugs, dosage, route of administration, interaction with other compounds, and time of exposure (60, 61). In addition, antioxidants may not exhibit antimutagenic effects or even act as pro-oxidants (62–65). The literature regarding the effects of antioxidants is still controversial, so further investigations are necessary to better understand their interaction with meglumine antimoniate and its lack of dose-response effects.

In conclusion, since genistein and ascorbic acid, two recognized antioxidants, were able to reduce the DNA damage caused by meglumine antimoniate, the present data support that the antileishmanial induces oxidative stress-derived DNA damage by promoting overactivation of the SOD-CAT axis. Additionally, our data reinforce the protective potential of these two compounds against reactive oxygen species, opening up perspectives for novel therapeutic strategies to minimize the side effects caused by meglumine antimoniate, including protection against genotoxic damage.

MATERIALS AND METHODS

Animals. To evaluate the antigenotoxic effects of genistein and ascorbic acid on genetic damage induced by meglumine antimoniate, five Swiss *Mus musculus* mice (male, healthy, 30 g, 8 weeks old) were used per treatment group; they were obtained from the Central Bioterium of the Federal University of Maranhão (UFMA) (São Luís, Maranhão, Brazil). Animals were kept in polycarbonate boxes under controlled conditions: temperature around $21 \pm 2^\circ\text{C}$, 12-h light-dark cycle, and water and food *ad libitum*. All procedures were done according to guidelines of the Brazilian College of Animal Experimentation (COBEA) and were approved by the Animal Experimentation Ethics Committee (CEUA-UFMA, no. 23115.012909-2013-41).

Chemical agents. Meglumine antimoniate (Glucantime) was from the pharmacy of the University Hospital Presidente Dutra (HUUFMA) (batch 246888; Sanofi-Aventis, São Paulo, Brazil), genistein (4',5,7-trihydroxyisoflavone; molecular weight, 270.24 g/mol) was obtained from Sigma-Aldrich (St. Louis, MO, USA), and ascorbic acid was obtained from Dinâmica Química Contemporânea Ltd. (São Paulo, Brazil). For the positive control, we used the mutagen cyclophosphamide (batch 43H0269) obtained from Sigma-Aldrich (St. Louis, MO, USA).

Treatments and doses. The dosage for meglumine antimoniate was estimated from the maximum recommended dosage for treatment of patients with leishmaniasis, that is, 20 mg/Sb³⁺/kg/day for 20 days (16). Doses for cyclophosphamide, ascorbic acid, and genistein were based on work conducted by Cantanhêde et al. (27), Vijayalaxmi and Venu (21), and Pugalendhi et al. (22), respectively.

For this study, we opted for an acute treatment using 810 mg/kg body weight of meglumine antimoniate (16). To evaluate the occurrence of dose-dependent effects, we used three different doses of the antioxidants, as follows: ascorbic acid, 30 mg/kg, 60 mg/kg, and 120 mg/kg; genistein, 5 mg/kg, 10 mg/kg, and 20 mg/kg. The negative control (NC) group was administered distilled water. The vehicle control (VC) group received 1% dimethyl sulfoxide (DMSO), the same concentration used to dilute genistein. The positive control (PC) group was treated with a single dose of 50 mg/kg cyclophosphamide. The meglumine antimoniate group (GLU group) was treated with a single dose of 810 mg/kg meglumine antimoniate (Table 4).

To evaluate the protective effect of genistein, groups G1, G2, and G3 received 5 mg/kg, 10 mg/kg, and 20 mg/kg genistein, respectively, for three consecutive days. On the third day, animals received their respective doses of genistein together with 810 mg/kg of meglumine antimoniate. To evaluate the protective effect of ascorbic acid, three types of treatment were conducted: the pretreatment group (PRE) received a single dose of ascorbic acid (60 mg/kg) and 24 h later received a single dose of meglumine antimoniate (810 mg/kg); the posttreatment group (POS) received a single dose of meglumine antimoniate (810 mg/kg) and 24 h later received a single dose of ascorbic acid (60 mg/kg); and groups with simultaneous treatment (S1, S2, and S3) received simultaneously a single dose of 30 mg/kg, 60 mg/kg, and 120 mg/kg ascorbic acid, respectively, and 810 mg/kg meglumine antimoniate (Table 4).

Doses of meglumine antimoniate, cyclophosphamide, and ascorbic acid were administered intraperitoneally, standardized in solutions of 0.1 ml per 10 g body weight. Doses of genistein and 1% DMSO were administered via gavage.

Comet assay. For the comet assay (66, 67), after 24 h of treatment, peripheral blood (10 μl) was obtained and mixed with 100 μl low-melting-point agarose (0.5%). This mixture was then applied to

TABLE 4 Experimental groups and their respective treatments

Group	Chemical agent(s)	Dose (mg/kg)	Administration
Genistein			
VC	DMSO	1%	Gavage
PC	Cyclophosphamide	50	Intraperitoneal
GLU	Meglumine antimoniate	810	Intraperitoneal
G1	Genistein + meglumine antimoniate	5 + 810	Gavage + intraperitoneal
G2	Genistein + meglumine antimoniate	10 + 810	Gavage + intraperitoneal
G3	Genistein + meglumine antimoniate	20 + 810	Gavage + intraperitoneal
Ascorbic acid			
NC	Distilled water		
PC	Cyclophosphamide	50	Intraperitoneal
GLU	Meglumine antimoniate	810	Intraperitoneal
S1	Ascorbic acid + meglumine antimoniate	30 + 810	Intraperitoneal
S2	Ascorbic acid + meglumine antimoniate	60 + 810	Intraperitoneal
S3	Ascorbic acid + meglumine antimoniate	120 + 810	Intraperitoneal
PRE	Ascorbic acid + meglumine antimoniate	60 + 810	Intraperitoneal
POS	Meglumine antimoniate + ascorbic acid	810 + 60	Intraperitoneal

pregelled slides with high-melting-point agarose (1.5%). Slides, in duplicate, were covered with coverslips and refrigerated for 5 min. After solidification, slides were immersed in cold lysis solution (2.5 M NaCl, 100 mM Na₂EDTA, 10 mM Tris [pH 10], 10% DMSO, and 1% Triton X-100) and refrigerated for 12 h at 4°C. Slides were then incubated for 20 min in alkaline buffer (10 M NaOH, 0.2 M EDTA, and distilled water, pH 13), followed by electrophoresis for 20 min at 25 V (0.72 V/cm) and 300 mA. Slides were neutralized (0.4 M Tris-HCl, pH 7.5) for 15 min and fixed with absolute ethanol for 4 min. The slides were stained with ethidium bromide (20 µg/ml) and analyzed by fluorescence microscopy (BX51/BX52-Olympus; 516- to 560-nm filter, 40× lens). For each animal, we analyzed 100 nucleoids. Each nucleoid was classified according to DNA damage at five levels: class 0, no damage (<5%); class 1, low damage (5 to 20%); class 2, medium damage (21 to 40%); class 3, high damage (41 to 94%); and class 4, total damage (>95%). Damage scores were calculated by multiplying the number of nucleoids in each class by their respective class value (68).

The percentage of reduction in meglumine antimoniate-induced genotoxic damage was calculated as described by Waters et al. (69): percent reduction = $(\text{GLU} - \text{TG}/\text{GLU} - \text{NC}) \times 100$, where GLU corresponds to the average damage in the group treated solely with meglumine antimoniate, TG is the average damage observed in groups treated using genistein with meglumine antimoniate or ascorbic acid with meglumine antimoniate, and NC corresponds to the average damage observed in the negative control or vehicle control group.

Comet assay followed by Fpg enzyme. To evaluate oxidative damage to DNA caused by meglumine antimoniate, we also conducted a comet assay using formamide pyrimidine DNA glycosylase (Fpg) (Fpg Flare assay kit; Trevigen, Gaithersburg, MD) (70). After lysis in lysis solution (2.5 M NaCl, 100 mM EDTA, and 10 mM Tris [pH 10] with 10% dimethyl sulfoxide and 1% Triton X-100), slides were dipped in 1× Flare buffer (Trevigen) for 30 min. Each slide was then treated with 150 µl Fpg enzyme solution (0.5 U), covered with coverslips, and incubated at 37°C for 45 min in humid chamber. After incubation, the coverslips were carefully removed, and electrophoresis, neutralization, fixation, staining, and data analysis were conducted as described above.

Micronucleus test. For the micronucleus test (71), after blood collection for the comet assay, animals were euthanized by anesthetic overdose (1 ml/kg xylazine and 0.1 ml/kg ketamine), and bone marrow was extracted using 1 ml fetal bovine serum. The cell suspension was centrifuged for 5 min at 1,000 rpm. The supernatant was discarded, and cell pellet smearing was conducted. Slides were stained using the hematological dye Panoptic (Laborclin) to differentiate polychromatic erythrocytes (PCE), colored in light purplish blue, from normochromatic erythrocytes (NCE), which stain in light red.

To investigate the micronucleus, 2,000 polychromatic erythrocytes were analyzed for each animal in an optical microscope at a magnification of ×1,000 (Carl Zeiss Primo Star). The PCE/NCE ratio was calculated using 300 erythrocytes from each animal to estimate toxicity for cyclophosphamide, meglumine antimoniate, and meglumine antimoniate associated with genistein and ascorbic acid.

Statistical analysis. Data normality was assessed by the Kolmogorov-Smirnov test. Data with a normal distribution were tested by analysis of variance (ANOVA) followed by Tukey's *ad hoc* test. A *P* value of <0.05 was considered significant. The comparison between the damage scores obtained from both the comet assays (standard and Fpg version) was performed by the Student *t* test. All data were evaluated using GraphPad Prism 6.

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