Meglumine Antimoniate (Glucantime) Causes Oxidative Stress-Derived DNA Damage in BALB/c Mice Infected by *Leishmania* (*Leishmania*) *infantum*

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ABSTRACT Leishmaniasis is a neglected tropical disease caused by >20 species of the protozoan parasite *Leishmania*. Meglumine antimoniate (Glucantime) is the first-choice drug recommended by the World Health Organization for the treatment of all types of leishmaniasis. However, the mechanisms of action and toxicity of pentavalent antimonials, including genotoxic effects, remain unclear. Therefore, the mechanism by which meglumine antimoniate causes DNA damage was investigated for BALB/c mice infected by *Leishmania* (*Leishmania*) *infantum* and treated with meglumine antimoniate (20 mg/kg for 20 days). DNA damage was analyzed by a comet assay using mouse leukocytes. Furthermore, comet assays were followed by treatment with formamidopyrimidine-DNA glycosylase and endonuclease III, which remove oxidized DNA bases. In addition, the activities of superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) in the animals' sera were assessed. To investigate mutagenicity, we carried out a micronucleus test. Our data demonstrate that meglumine antimoniate, as well as *L. infantum* infection, induces DNA damage in mammalian cells by the oxidation of nitrogenous bases. Additionally, the antileishmanial increased the frequency of micronucleated cells, confirming its mutagenic potential. According to our data, both meglumine antimoniate treatment and *L. infantum* infection promote oxidative stress-derived DNA damage, which promotes overactivation of the SOD-CAT axis, whereas the SOD-GPx axis is inhibited as a probable consequence of glutathione (GSH) depletion. Finally, our data enable us to suggest that a meglumine antimoniate regimen, as recommended by the World Health Organization, would compromise GPx activity, leading to the saturation of antioxidant defense systems that use thiol groups, and might be harmful to patients under treatment.

KEYWORDS antileishmanial, antimony, genotoxicity, mutagenicity, antioxidant enzymes

Leishmaniasis is a neglected tropical disease caused by >20 species of the protozoan parasite *Leishmania* (*Trypanosomatidae*), which are transmitted by infected female phlebotomine sandflies. Wild and domesticated animals, and humans themselves, can act as reservoirs of infection. There are three main forms of the disease: (i) cutaneous leishmaniasis (CL), the most common form, which causes skin lesions; (ii) mucocutaneous leishmaniasis (MCL), which leads to partial or total destruction of mucous

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membranes; and (iii) visceral leishmaniasis (VL), which is fatal if not treated appropriately. It is characterized by irregular fever, weight loss, enlargement of the spleen and liver, and anemia (1).

In 2014, >90% of new leishmaniasis cases occurred in six countries: Brazil, Ethiopia, India, Somalia, South Sudan, and Sudan. In fact, visceral leishmaniasis is widely distributed around the world and is a public health problem in 88 countries. Moreover, 310 million people are at risk of infection in six countries that report >90% of VL cases worldwide. Recent epidemiological data show that of the 25 countries with high leishmaniasis burdens, 13 have high burdens of VL only, 11 have high burdens of CL only, and 1 (Brazil) has high burdens of both VL and CL. Although many people have asymptomatic infections in areas of VL endemicity, the risk of developing active infection is dramatically increased for HIV-positive patients (2).

Meglumine antimoniate (Glucantime) is a pentavalent antimony (Sb^{V}) recommended by the World Health Organization as the first-choice drug for the treatment of all types of leishmaniasis; the maximum dose recommended is 20 mg/kg of body weight/day via the intramuscular route, although many side effects are still reported. The most frequently reported clinical adverse effects are musculoskeletal pain, nausea, vomiting, diarrhea, abdominal pain, headache, anorexia, asthenia, fatigue, fever, exanthema, erythema, and urticaria. Moreover, patients may develop cardiac, liver, and kidney complications, usually at the end of the treatment (3). Recently, the occurrence of patients with drug resistance has been reported more often, and death by bleeding in northeastern Brazil (e.g., in Maranhão State) has been reported (unpublished data).

Although pentavalent antimonials have been used for >6 decades, the pharmacological and toxicological mechanisms involved in their action remain unclear. Berman et al. (4) and Demicheli et al. (5) proposed that this drug interferes with the bioenergetic process of *Leishmania* amastigotes, forming stable complexes with ribonucleosides, which interfere with the parasite's fatty acid β -oxidation and glycolysis, promoting the depletion of intracellular ATP levels. Another hypothesis suggests that Sb^{V} acts as a prodrug that is transformed into the more toxic trivalent form (Sb^{III}) to exert its antileishmanial activity (6–9). In fact, some studies indicate that Sb^{III} production *in vivo* is responsible for both therapeutic activity and antimony toxicity (10–12). Furthermore, we have confirmed this hypothesis by showing that meglumine antimoniate causes DNA damage *in vivo* but not *in vitro*. This suggests that Sb^{V} is a promutagen that requires metabolization to exert its mutagenic action (13).

There is evidence that Sb^{III} has the ability to change the cellular redox potential, leading to intracellular thiol efflux and trypanothione reductase inhibition (8, 9, 14). Furthermore, studies have shown that antimonial compounds increase levels of reactive oxygen species (ROS)/reactive nitrogen species (RNS), altering enzyme activities related to the antioxidant defense system (15–17).

Considering our previous reports that demonstrated the capacity of meglumine antimoniate to induce mutations *in vivo* (13), the frequency of which was reduced by antioxidant isoflavones (18), in the present study we investigated whether the mutagenic effect of meglumine antimoniate is related to its ability to promote oxidation in DNA nitrogenous bases by oxidative stress.

RESULTS

***Leishmania* infection and meglumine antimoniate cause genomic damage by the oxidation of DNA bases.** Figure 1A and B present the frequencies of nucleoid classes observed in peripheral blood leukocytes from BALB/c mice infected with *Leishmania infantum* and/or treated with meglumine antimoniate, as determined by conventional comet assays and by comet assays followed by digestion with formamidopyrimidine-DNA glycosylase (Fpg) and endonuclease III (Endo III). The distribution of nucleoids in all experimental groups, infected or uninfected, receiving meglumine antimoniate differed from that in the negative-control group such that classes with higher damage scores appeared. This new distribution of damage classes is reflected in the DNA damage scores (Fig. 1C). In the conventional comet assay (Fig. 1A), G1 animals, which were infected

FIG 1 (A and B) Frequencies of nucleoid classes in peripheral blood leukocytes from different groups of BALB/c mice infected with *Leishmania (Leishmania) infantum* and treated with meglumine antimoniate (n , 5 per group), observed by use of a conventional comet assay (A) or by a comet assay followed by Fpg and Endo III digestion (B). (C) DNA damage scores (means + standard deviations) for different groups. NC, negative control (saline); G1, animals infected with *Leishmania (Leishmania) infantum* for 65 days; G2, uninfected animals treated with meglumine antimoniate for 20 days (20 mg/kg/day); G3, infected animals receiving meglumine antimoniate for 20 days (20 mg/kg/day). Asterisks indicate statistical differences (*, $P < 0.05$; **, $P < 0.001$; ***, $P < 0.0001$) by ANOVA followed by a Tukey test.

but not treated with the antimonial, presented a higher damage score (1.08 ± 0.21) than negative-control animals (0.35 ± 0.08) ($P < 0.01$), showing that infection *per se* was capable of causing DNA damage. Similarly, G2 animals, which were not infected but received meglumine antimoniate at 20 mg/kg/day for 20 days (the same prescription recommended for humans), showed significant increases in genomic lesions (1.31 ± 0.36) over the negative-control group ($P < 0.01$), confirming the genotoxic effect of meglumine antimoniate. In addition, score data showed that infection and meglumine antimoniate cause similar levels of DNA damage ($P > 0.05$). Moreover, infected animals treated with meglumine antimoniate for 20 days (G3) also presented a damage score (0.84 ± 0.17) significantly different from that of negative-control animals ($P < 0.05$). However, G3 animals presented DNA damage scores lower than those of uninfected animals receiving meglumine antimoniate. On the other hand, their damage scores did not differ from those of G1 animals, which had only *Leishmania* infection.

After enzymatic digestion, no class 0 nucleoids were observed in any of the experimental groups (Fig. 1B). Moreover, the frequencies of the higher-damage classes (classes 3 and 4) increased dramatically (Fig. 1B), revealing higher damage scores than those obtained by the conventional comet assay (Fig. 1C). This increase in DNA damage after the action of Fpg and Endo III enzymes suggests that *Leishmania* infection *per se*, as well as meglumine antimoniate, causes genomic damage by the oxidation of DNA bases.

Meglumine antimoniate increases the frequency of mutations. Cytotoxicity was determined by calculating the ratio of polychromatic erythrocytes (PCE) to normochromatic erythrocytes (NCE) using 1,000 erythrocytes per animal. None of the groups treated with meglumine antimoniate showed any difference in cytotoxicity from the

TABLE 1 Frequency of micronucleated polychromatic erythrocytes and PCE/NCE ratio in the bone marrow of BALB/c mice infected with *Leishmania* (*Leishmania*) *infantum*

Group ^a	%o MnPCE (mean no. per animal) ^b	PCE/NCE ratio ^c
NC	23 (3.0) A	1.04
G1	76 (9.2) AB	0.94 (NS)
G2	104 (15.2) B	1.09 (NS)
G3	105 (15.0) B	1.07 (NS)

^aNC, negative control (saline); G1, animals infected with *L. infantum* for 65 days; G2, uninfected animals treated with meglumine antimoniate for 20 days (20 mg/kg/day); G3, infected animals receiving meglumine antimoniate for 20 days (20 mg/kg/day).

^bMnPCE, micronucleated polychromatic erythrocytes detected per group. There were 5 animals per group, and 2,000 erythrocytes per animal were tested, for a total of 10,000 erythrocytes per group. Different letters indicate statistical differences by the Kruskal-Wallis test followed by Dunn's test ($H = 13.9055$; $P = 0.0030$).

^cPCE, polychromatic erythrocytes; NCE, normochromatic erythrocytes; NS, not significant (there is no difference from the result for the negative control).

negative-control group (Table 1). With regard to mutagenicity, there was no difference between the infected group (G1) and the negative-control group, indicating that the infection *per se* is not capable of causing mutations. On the other hand, all groups receiving meglumine antimoniate (G2 and G3) presented a higher frequency of micronucleated cells than did the negative-control group. In addition, there was no difference between uninfected (G2) and infected (G3) animals treated with meglumine antimoniate for 20 days (Table 1).

Meglumine antimoniate interferes with the activities of antioxidant enzymes.

To further assess the animals' serum antioxidant defense statuses, the activities of superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) were determined. *Leishmania* infection (G1) increased the level of SOD activity over that in negative-control mice, and this effect was potentiated by meglumine antimoniate treatment (G2 and G3) (Fig. 2A). Similarly, in all experimental groups, CAT activity was

FIG 2 Activities of the antioxidant enzymes (means + standard deviations) superoxide dismutase (A), catalase (B), and glutathione peroxidase (C). NC, negative control (saline); G1, animals infected with *L. infantum* for 65 days; G2, uninfected animals treated with meglumine antimoniate for 20 days (20 mg/kg/day); G3, infected animals receiving meglumine antimoniate for 20 days (20 mg/kg/day). Different letters above the bars indicate statistical differences ($P < 0.05$) determined by ANOVA followed by a Newman-Keuls test.

significantly increased over that for the negative control, but no difference was found among experimental groups (Fig. 2B). On the other hand, GPx activity was significantly reduced in all experimental groups from that in the negative-control group (Fig. 2C). In this case, meglumine antimoniate *per se* (G2) apparently caused less of a reduction in GPx activity than infection (G1). Hence, this set of data emphasizes that both *Leishmania* infection and meglumine antimoniate treatment disturb the serum redox balance, which, in turn, distinctly modulates the activity of antioxidant enzymes.

DISCUSSION

Previously, our group demonstrated the genotoxic effects of meglumine antimoniate on the DNA of uninfected Swiss mice receiving a single dose of the drug (13), as well as the ability of antioxidants to prevent these effects (18). Based on those findings, this study was conducted on BALB/c mice infected with *Leishmania* (*Leishmania*) *infantum* in order to investigate the mechanism by which meglumine antimoniate causes DNA lesions.

Our results, presented here, demonstrated the ability of meglumine antimoniate (20 mg/kg/day) given for 20 days to cause DNA damage in mice, confirming the results obtained by Lima et al. (13), who demonstrated that meglumine antimoniate induces genomic instability *in vivo* but not *in vitro*, suggesting that meglumine antimoniate is a promutagen. These data support the “prodrug model of antimony action” proposed by Goodwin and Page (19), who firstly suggested that the pentavalent antimony is converted into the more toxic trivalent form to exert its antileishmanial activity. Sereno et al. (20) also demonstrated the ability of Sb^{III} at low concentrations to induce DNA fragmentation in *Leishmania infantum* amastigotes.

To elucidate how meglumine antimoniate interacts with DNA, we carried out a comet assay followed by enzymatic digestion. This assay is based on the fact that the bacterial enzymes formamidopyrimidine-DNA glycosylase (Fpg) and endonuclease III (Endo III) recognize different types of oxidative damage. Fpg is specific for oxidized purines, including 8-oxo-7,8-dihydroguanine (8-oxoGua), 2,6-diamino-4-hydroxy-5-formamidopyrimidine (FaPyGua), and 4,6-diamino-5-formamidopyrimidine (FaPyAde) and other ring-opened purines. Endo III recognizes oxidized pyrimidines, including thymine glycol and uracil glycol. Consequently, both enzymes have been used as biomarkers of DNA damage caused by oxidative stress (21–24). Our results showed that enzymatic digestion further increased genomic lesions caused by meglumine antimoniate, suggesting that DNA damage was induced by the oxidation of the nitrogenous bases of DNA. This result is consistent with the fact that Sb^{III} is a borderline metal ion with a high affinity for nitrogen- and sulfhydryl-containing ligands (25). Our results also support the findings of Cantanhêde et al. (18), who showed that soybean isoflavone, a potent antioxidant, was able to reduce the genetic damage caused by meglumine antimoniate.

ROS/RNS cause damage to cell macromolecules, including lipids, proteins, and DNA. Cavallo et al. (26) demonstrated the occurrence of oxidative DNA damage in workers exposed to antimony trioxide. It was also shown that Sb^{III} increases OH[•] and H₂O₂ levels, inducing apoptosis through an increase in ROS levels (27, 28). Other studies suggest that both arsenic and antimony are responsible for inducing oxidative stress through the depletion of reducing agents, such as glutathione (GSH) (29, 30). More recently, Bento et al. (17) demonstrated that meglumine antimoniate causes protein carbonylation in the heart, spleen, and brain, besides lipid peroxidation in the livers and brains of CF-1 mice. Additionally, Kato et al. (31) reported higher peroxidase enzyme activity in animals treated with meglumine antimoniate. Hence, our data support the idea that the toxicity of antimonials is related to the production of reactive oxygen species.

Our data also revealed that *L. infantum* infection *per se* induces genomic lesions. Other studies reported that parasites such as *Trypanosoma cruzi*, *Toxoplasma gondii*, and *Opisthorchis viverrini* also induce DNA damage (32–35). Our results are also consistent with those of Kocyigit et al. (36) and de Oliveira et al. (37), who observed DNA

damage in patients with cutaneous leishmaniasis and in mice infected by *Leishmania chagasi*, respectively. These authors related the genotoxic effect to elevated NO generation during the control of *Leishmania* infection through the process known as the "oxidative burst," causing DNA damage in host cells by nitrative and oxidative lesions. Our data confirmed this hypothesis once we demonstrated that infection with *L. infantum* causes genomic instability through the oxidation of DNA bases.

Considering that the comet assay detects genomic lesions, we carried out a micronucleus test to determine whether these primary lesions caused by oxidative damage would be fixed through DNA repair mechanisms. Our data showed that *Leishmania* infection itself was not able to increase the frequency of micronucleated cells over that for the negative control, revealing that although the infection has genotoxic action, it does not induce mutations. Accordingly, the DNA repair system was able to remove oxidized bases induced by *Leishmania* infection. On the other hand, there was a higher frequency of micronucleated cells in animals treated with meglumine antimoniate. These data are consistent with those of Gebel (38) and Gurnani et al. (39), who reported that high antimony levels induced higher frequencies of micronuclei, DNA double-strand breaks, and chromosomal aberrations in mice. Additionally, Hei et al. (40) reported that arsenic, which has chemical and toxicological characteristics similar to those of antimony, caused mutations in mammalian cells mediated by ROS generation. More recently, Lima et al. (13) described the mutagenic effect of meglumine antimoniate in Swiss mice receiving acute doses of the drug. Additionally, Cantanhêde et al. (18) reported the ability of antioxidants to reduce the mutagenic effect of meglumine antimoniate.

The antioxidant defense system, which plays an important role in redox homeostasis maintenance, is composed of three main antioxidant enzymes, i.e., superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx), whose ability to remove reactive oxygen intermediates is crucial to maintaining redox balance. SOD rapidly dismutates $O_2^{\cdot-}$ into H_2O_2 , which is further reduced to H_2O by both CAT and GPx, besides peroxiredoxins. Otherwise, in the presence of Fe^{2+} , H_2O_2 undergoes the Fenton reaction to generate the superoxidizing hydroxyl radical ($OH^{\cdot}$) (41, 42).

Taking this into account, we assessed the activities of SOD, CAT, and GPx in the sera of animals from groups G1, G2, and G3. Our results demonstrated that *Leishmania* infection itself increased SOD activity, possibly as an attempt to compensate for $O_2^{\cdot-}$ overproduction. These data are consistent with those of Britti et al. (43) and Souza et al. (44), who observed higher SOD activity in *Leishmania infantum*-infected dogs. Similarly, Khouri et al. (45) described increased SOD activity in patients presenting with cutaneous leishmaniasis. These authors considered SOD1 plasma levels a useful tool for distinguishing clinical status and predicting the failure of Sb^V treatment. On the other hand, they reported a decrease in plasma SOD1 levels after antimony therapy (20 mg/kg/day for 20 days). Britti et al. (46) observed no significant difference in SOD activity in dogs with leishmaniasis upon similar therapy. It is noteworthy that our results also showed an increase in SOD activity for animals receiving only meglumine antimoniate, suggesting that this antileishmanial increases SOD activity regardless of infection. These data confirm the results of López et al. (47), who found increased SOD activity in red blood cells exposed to pentavalent antimony *in vitro*.

In accordance with the SOD activity data, either *Leishmania* infection or meglumine antimoniate also increased CAT activity. Previous reports have found high CAT levels in *Leishmania*-infected animals (48), while others found lower enzymatic activity (47). Notwithstanding, Bento et al. (17), studying uninfected animals receiving meglumine antimoniate, observed that CAT activity differs according to the organ evaluated. Once again, according to our data, *Leishmania* infection and meglumine antimoniate seem to independently promote oxidative stress, probably by acting on the same pathway, since there was no difference in the enzymes' activities when both conditions were present concomitantly.

Our data showed that serum GPx activity in all groups decreased from that for the negative control. These data are consistent with those of Souza et al. (44), who observed lower GPx activity in blood and plasma from *Leishmania infantum*-infected

dogs. On the other hand, Britti et al. (46) observed no difference in GPx activity in infected dogs after 60 days of therapy with *N*-methylglucamine antimoniate. Besides, it has been shown that GPx activity is directly proportional to free GSH concentrations (49). According to Singh et al. (50), high ROS production, such as that observed in the oxidative burst, may lead to saturation of this antioxidant pathway due to the large consumption of GSH by GPx. These observations are in line with our results, especially because it has been shown that Sb^{III} is able to impair the redox balance by promoting intracellular GSH depletion and glutathione reductase inhibition, thereby preventing GSH regeneration (14–18).

With regard to the bench-to-bedside relevance of our data, we must consider that dose extrapolation among species depends on characteristics such as the animal's anatomy, physiology, and biochemical processes, as well as drug pharmacokinetics, for proper allometric scaling (51). It should be noted that the animal dose used in this work was equivalent to approximately 1.5 mg/kg/day in humans, much lower than the regimen recommended by the WHO for humans, i.e., 20 mg/kg/day for 20 days (52). Nevertheless, the dose we used was able to cause oxidative stress-derived DNA damage, corroborating previous studies that demonstrated lipid peroxidation and protein carbonylation in animals receiving 20, 60, or 120 mg/kg/day of meglumine antimoniate for 3 days, with no difference in the resultant oxidative damage among doses (17).

Considering that the regimen of treatment used in this study is below the WHO's guidelines (according to the animal equivalent dose calculation), the genomic instability might be deeper when higher doses are used, as in the human therapy. In addition, considering the correlation between mutagenicity and carcinogenicity (53), exposure of humans to mutagens should be avoided whenever possible. Therefore, studies for estimating the risk to patients under treatment must be performed.

In summary, the data presented here confirm the mutagenic effect of meglumine antimoniate, previously reported in uninfected animals by our group (13). Moreover, to the best of our knowledge, this work reports for the first time that meglumine antimoniate *per se*, as well as *Leishmania infantum* infection, is a DNA stressor, causing oxidation of the nitrogenous bases of DNA. Nevertheless, the mechanisms of action of *Leishmania* (*Leishmania*) *infantum* infection and meglumine antimoniate do not seem to be associative, since the concomitant presence of both stressors did not enhance DNA damage. According to our data, these stressors promote oxidative stress-derived DNA damage, which caused overactivation of the SOD-CAT axis, whereas the GPx branch was inhibited as a probable consequence of GSH depletion.

MATERIALS AND METHODS

Animals. BALB/c mice were kept for many generations in the Animal Breeding Unit of the University of Campinas (Sao Paulo, Brazil) under standard conditions. They were kept in a well cross-ventilated room at $26 \pm 2^\circ\text{C}$ with a relative humidity of 44 to 56% and light-dark cycles of 12 h. The animals had free access to sterilized food and water. Five 8-week-old male mice weighing 30 g were used for each experimental group. All international, national, and/or institutional guidelines that were applicable for the care and use of animals were followed. All procedures were in accordance with the requirements of COBEA (Brazilian College of Animal Experimentation). The project was approved by the Ethics Committee for Animal Experimentation (CEUA), with protocol number 23115.012909-2013-41.

Parasites. *Leishmania* (*Leishmania*) *infantum* promastigotes (MHOM/BR/1970/BH46) were grown to stationary phase using Schneider medium (Sigma, St. Louis, MO, USA) supplemented with 10% inactivated fetal bovine serum (Gibco). The parasites were maintained at 27°C until injection into animals.

Infection and treatment. *Leishmania* (*Leishmania*) *infantum* stationary-phase promastigotes ($10^7/50 \mu\text{l}$ of saline) were subcutaneously injected into the right hind footpad. The infection was maintained for 65 days in all experimental groups. On the 66th day, treatments were carried out for the following groups: NC, negative control (saline); G1, infection control (infected animals with no further treatment); G2, uninfected animals treated with meglumine antimoniate for 20 days (20 mg/kg/day); G3, infected animals receiving meglumine antimoniate for 20 days (20 mg/kg/day). The drug was obtained from the Brazilian Ministry of Health (meglumine antimoniate, batch 246888; Sanofi-Aventis, São Paulo, Brazil) and was administered intraperitoneally in volumes of 0.1 ml/10 g of body weight. The dose was determined by considering data reported by Bento and collaborators (17), as well as the regimen recommended by the WHO for treating leishmaniasis patients, i.e., 20 mg Sb^V/kg/day for 20 days (52).

Comet assay. The comet assay was based on the work of Singh et al. (54) and Tice et al. (55). On the 21st day from the beginning of treatment, peripheral blood leukocytes were obtained by excising the end of the tail of each animal. The cell pellet was added to 200 μl of low-melting-point agarose (0.5%),

which was distributed in pregelated slides with normal-melting-point agarose. After cooling for 10 min, slides were immersed in a lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 10% dimethyl sulfoxide, 1% Triton X-100 [pH 10.0]) and were refrigerated at 4°C overnight. Slides were incubated for 20 min in an alkaline buffer (10 M NaOH, 0.2 M EDTA, and distilled water [pH 13]), followed by electrophoresis at 25 V and 300 mA for 25 min. Slides were neutralized (0.4 M Tris-HCl [pH 7.5]) for 15 min, dried at room temperature, and fixed in 100% ethanol. They were stained with ethidium bromide (20 µg/ml) and were analyzed under an Olympus BX51/BX52 fluorescence microscope (516- to 560-nm filter; 590-nm barrier filter; 40× lens). One hundred nucleoids were analyzed by treatment, taking into account both the size and the amount of DNA in the comet tail. DNA damage was classified into 5 levels: class 0, no damage (<5%); class 1, a low level of damage (5 to 20%); class 2, a medium level of damage (21 to 40%); class 3, a high level of damage (41 to 94%); class 4, total damage (>95%). Damage scores were calculated by multiplying the number of nucleoids in each class by the respective class value (56).

Comet assay followed by Fpg and Endo III digestion. In order to assess DNA oxidative damage, a comet assay modified for the detection of oxidized bases using the repair endonucleases Endo III (endonuclease III) and Fpg (formamidopyrimidine-DNA-glycosylase) was performed to detect oxidized pyrimidines and purines, respectively (57). Briefly, after the lysis step, slides were soaked in 1× FLARE (fragment length analysis using repair enzymes) buffer (Trevigen) for 30 min. Then each slide was treated with 150 µl of a solution containing Fpg and Endo III enzymes, covered with coverslips, and incubated at 37°C for 45 min in a humid chamber. After the incubation period, coverslips were carefully removed, and electrophoresis, neutralization, fixing, staining, and data analysis were conducted as described above.

Micronucleus test. The micronucleus test was conducted according to the method of Schmid (58). Animals were sacrificed by anesthetic overdose (1 ml/kg xylazine and 0.1 ml/kg ketamine), and the bone marrow of one femur was extracted using 500 µl fetal bovine serum and was centrifuged at 1,000 rpm for 5 min. The supernatant was discarded, and cell pellet smearing was conducted. The material was dried at room temperature, and cells were stained using a fast hematologic dye (Panotic; Laborclin). The resulting color distinguishes between polychromatic erythrocytes (PCE), which stain light purplish blue, and normochromatic erythrocytes (NCE), which stain light red.

The frequency of micronucleated cells was determined with 2,000 polychromatic erythrocytes per animal, while toxicity was established by the PCE/NCE ratio in 1,000 erythrocytes per animal.

SOD activity. Total superoxide dismutase (SOD) activity was determined by use of a superoxide dismutase assay kit (Cayman Chemical, Ann Arbor, MI, USA) in serum (10 µl) according to the manufacturer's instructions. On a 96-well plate, the reaction was initiated by adding 20 µl of xanthine oxidase in 10 µl of serum. The plate was incubated on a shaker at room temperature for 20 min, and absorbance was measured at 450 nm using a plate reader (Synergy H1; BioTek).

CAT activity. The catalase (CAT) assay was carried out by use of a catalase assay kit (Cayman Chemical, Ann Arbor, MI, USA) according to the manufacturer's instructions. On a 96-well plate, the reaction was initiated by the addition of 20 µl hydrogen peroxide in 20 µl of serum, with subsequent incubation on a shaker at room temperature for 20 min. Then 30 µl potassium hydroxide was added to complete the reaction. Thirty microliters of catalase was added, and the solution was incubated again on a shaker at room temperature for 10 min. Subsequently, 10 µl potassium periodate catalase was added to each well, and the solution was incubated for 5 min at room temperature. Absorbance was measured at 540 nm using a plate reader (Synergy H1; BioTek).

GPx activity. Glutathione peroxidase (GPx) activity was determined using a glutathione peroxidase assay kit (Cayman Chemical, Ann Arbor, MI, USA) according to the manufacturer's instructions. In order to conduct the assay, 20 µl serum was used. On a 96-well plate, 20 µl cumene hydroperoxide was added in 20 µl serum, and the mixture (cumene hydroperoxide and serum) was carefully homogenized for 5 min. Absorbance was measured at 340 nm using an enzyme-linked immunosorbent assay (ELISA) plate reader (Synergy H1; BioTek).

Statistical analysis. Data normality was assessed by a Kolmogorov-Smirnov test. The parametric one-way analysis of variance (ANOVA) test was applied, followed by the Tukey test, for the comet assay. The ANOVA test was followed by the Newman-Keuls test for enzyme activities ($P < 0.05$). Results that did not have a normal distribution were assessed by a Kruskal-Wallis test, followed by a Student-Newman-Keuls test. Graphs were made using GraphPad Prism, version 6.

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