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Genotoxic Effects of Oral Ascorbate in Healthy Mice: Evaluation of DNA Single-strand Breaks and Micronucleus

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Abstract Ascorbate is part of the first-line antioxidant defense in biological fluids. It reacts with a variety of oxidants and is currently used in treatments to mitigate some diseases including cancer. However, this agent may cause DNA damage in healthy animal cells but the effects of ascorbate *per se* on biological systems have not yet been reported. Therefore, the aim of this study was to explore the ascorbate cytotoxic and genotoxic effects in cells from healthy mice (35 individuals in total, divided into 7 groups of 5 animals each and oral daily doses for 28 days). The results showed that ascorbate (at doses of 100, 150 and 225 mg/kg) causes a significant increase in micronuclei (MN) frequency (during the 4 weeks of administration: respectively 3, 4, 4 MN for every 2,000 reticulocytes, with a p value ≤ 0.05). The Pearson correlation index showed a positive and significant correlation $r^2= 0.9230$; $p= 0.0254$) between the highest dose (225 mg) and the time of administration. The lowest dose that was administered in this study (50 mg/kg) did not produce MN however, a significant increase of DNA single-strand breaks was observed during all experimental time (in average, the migration index was 1.28 in comparison to 1.1 of the control group, with a p value ≤ 0.05). Cell death by cytotoxicity was not observed at any dose. These results indicate that the administration of ascorbate in healthy subjects is not innocuous and that its administration should be supervised because nowadays, the supplementation of large amounts of ascorbate without any medical supervision is frequent.

Keywords: *ascorbate, cell viability, retyculocytes, DNA damage, genotoxicity*

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1. Introduction

The six-carbon lactone known as vitamin C, ascorbate or ascorbic acid is an essential micronutrient required for a large variety of functions. In biological fluids, this hydrosoluble antioxidant might eliminate antioxidant derived radicals and reactive oxygen and nitrogen species. In addition, ascorbate is part of the first-line antioxidant defense in biological systems [1].

Nowadays, self-administration of large doses of supplements that are considered innocuous, such as vitamins, which can be acquired by anyone without prescription at any drugstore is a generalized habit [2]. Regarding ascorbate, this conduct is supported and encouraged by reports about the benefits of administrating large doses of ascorbate to cure cancer or to ameliorate symptoms of the common cold [3]. Studies in humans indicate the benefits of ascorbate consumption reporting a decrease in heart and vascular diseases, coronary risk, protection against oxidative stress and lipid peroxidation in populations such as smokers or dwellers in heavily polluted cities. Epidemiological studies have suggested that in cardiovascular diseases, a decrease in the incidence and mortality is observed if ascorbate supplementation increases [1]. In addition, Enstrom [4] has reported that the increase in ascorbate consumption decreases the incidence and mortality in diseases associated with oxidative stress such as cancer and atherosclerosis as well as decrease of the oxidative damage observed in smokers [5] due to its antioxidant properties; some authors suggest that ascorbate might increase cellular repair capacity in the cells evaluated [6,7]. Ascorbate is marketed as a nutritional supplement in doses as high as 2,000 mg per day. Nowadays, any reader can access information on line about ascorbic acid, for instance, MedlinePlus [8] (a service of the US National Library of Medicine National

Institutes of Health) states that the consumption of ascorbate is probably an effective way to improve iron absorption and tyrosinemia; and possibly effective to ameliorate age-related macular degeneration, albuminuria, atherosclerosis, cancer, common cold, complex regional pain syndrome, erythema, gall bladder disease, hemolytic anemia, high blood pressure, lead poisoning, osteoarthritis, sunburn, among other pathologies.

In 2015, The American Cancer Society (ACS) reported that dietary supplements (including vitamins such as ascorbate) can have side effects. Since then, the number of reports of adverse reactions has increased yearly because people do not always follow the recommended dosage instructions or ingest supplements without medical supervision.

Although ascorbate is one of the most consumed [2] vitamins in the world, very little research about the effects produced on the main biomolecules such as the DNA of healthy individuals has been done. The experimental evidence obtained from healthy individuals is controversial, such is the case of the report [9] that states that administrating ascorbate (500 mg/day) to healthy people produces pro-oxidant and antioxidant effects. When this vitamin is administered with copper or iron, it results in the presence of oxidative biomarkers of lipids, proteins, and DNA [10].

The use of the cell viability technique in peripheral blood leukocytes to evaluate the cytotoxic potential of agents is very helpful because it has two advantages; first, it is a simple way to evaluate systemic cytotoxicity and secondly, it allows taking samples of the same individual over time since it is a non-invasive test. Currently, the micronucleus test and the alkaline single cell electrophoresis assay are part of the battery of techniques that are widely used for the identification of agents with genotoxic activity.

An increase in micronuclei frequency is an unequivocal indicator of genetic damage [11], this a universally valid test [12] used to identify important data to calculate cancer risk such as chromosomal loss or non-disjunction events caused by the action of microtubule inhibitors [13], which indicates that MN can be of clastogenic or aneuploidogenic origin.

The comet assay reveals breaks in the DNA strands in any nucleated cell [14]. The alkaline version (pH 13) of this technique, is the most versatile because it can detect DNA single or double strand breaks (SSB or DSB), alkali labile sites, crosslinks DNA-DNA or DNA-protein, and SSB associated with incomplete excision repair sites [15].

This also applies for the cell viability by fluorochromes, the same individual can be sampled over time because it's a non-invasive technique. Furthermore, the observed results correspond almost in real time with what happens in the body.

Nowadays, there are lots of epidemiological and experimental evidence showing that consumption of ascorbic acid helps mitigate the adverse effects of many diseases. However, the available literature regarding the adverse effects of ascorbate on the DNA of healthy subjects is almost inexistent.

Therefore, it is relevant to evaluate genotoxicity in agents that are regularly consumed by the population without any restrictions, and from which genotoxicity was not properly evaluated in the past or, as in the case of ascorbic acid, that inconclusive results have been reported in the literature. For this reason, we decided to evaluate the genotoxic and cytotoxic effects of orally administered ascorbate in healthy mice cells.

2. Materials and Methods

Animals. Thirty-five male CD-1 mice (60 days old, body weight 30 ± 2 g) were purchased from the vivarium of the Faculty of Medicine, UNAM. Animals were allowed to adjust to the laboratory conditions (22 ± 2 °C, humidity 57% and controlled lighting 12:12 light/dark regime) for 6 days. During all experimental time they received commercial standard mouse food (Purina Rodent Chow, Purina rodent Laboratory Chow, Mexico City, Mexico) and ad libitum drinking water. Once the mice adjusted to these conditions, they were randomly clustered into 7 groups (0, 50, 100, 150 and 225 mg/kg/day of ascorbic acid, colchicine and methyl methanesulfonate) with 5 animals per group. In this work, the experimental protocols were in accordance with the Guide for Care and Use of Laboratory Animals from the Institute of Laboratory Animal Resources Commission on Life Sciences National Research Council (2010) and the Mexican official norm NOM-062-ZOO-1999 for the production, care, use and sacrifice of laboratory animals. The Research and Ethical Committee from the Faculty of Medicine, UNAM, approved the project (095/2011).

Chemicals. The main chemicals reagents were purchased from Sigma-Aldrich Chemicals Company (St. Louis, MO, USA): Fluorescein-diacetate (CAS 1314-62-1), acetone (CAS 67-64-1), ethidium bromide (CAS 1239-45-8), sodium chloride (CAS 7647-14-5), acridine orange (CAS 10127-02-3), sodium phosphate dibasic anhydrous (CAS 7558-79-4), potassium dihydrogen phosphate (CAS 7778-77-0), methyl methanesulfonate (CAS 66-27-3), colchicine (CAS 64-86-8), low melting point agarose (CAS 39346-81-1), regular agarose (CAS 9012-36-6), TRIS hydrochloride (CAS 1185-53-1), EDTA (60-00-4), triton X-100 (CAS 9002-93-1), dimethyl sulfoxide (CAS 67-68-5), sodium hydroxide (CAS 1310-73-2), absolute ethanol (CAS 64-17-5). Redoxon® Bayer (trade name of a commercial ascorbic acid) was acquired from Bayer (Mexico).

2.1. Experimental Groups and Sampling

Ascorbate: The ascorbic acid was dissolved in drinking water (vehicle). For 28 days (4 weeks), a daily oral dose was administered to each animal through the orogastric tube. The specific dosage administered depended on the assigned group (0, 50, 100, 150 and 225 mg/kg). All animals were sampled at time zero (T0) and then, every 7 days for 4 weeks.

Genotoxicity positive controls: The aneugenic agent, colchicine (Col), was orally administered in a single dose of 5 mL/kg. This dose was selected in accordance with those reported by Schneider et al. [16]. The clastogenic agent, methyl methanesulphonate (MMS), was administered by intraperitoneal injection in a single dose of 40 mg/kg. This dose was administered in accordance with the report of Sugiyama et al. [17].

Sampling: 10 µL of whole blood were obtained by cutting the caudal vein of each mouse. Each blood sample was processed for cell viability by fluorochromes and micronuclei test with acridine orange. The samples from

groups 0 and 50 mg/kg were also processed for alkaline comet assay (pH 13).

The blood samples of the animals of the genotoxicity positive controls (Col and MMS) were taken at T0 and 48 h after the administration of the drug.

2.2. Experimental Techniques

Dual fluorochrome-mediated viability assay in peripheral blood leukocytes

The viability assay was performed with some modifications, as described by Rivera et al. [18]. Briefly, we´ll describe the technique. For stock solutions, fluorescein diacetate (FDA) was diluted 0.005 g/mL in acetone and ethidium bromide (Et-Br) was diluted 0.003 g/mL in phosphate buffer (PBS, pH 7.4). Then, in an amber recipient, 20 µL of FDA stock solution and 50 µL of Et-Br stock solution were added to 1.2 mL of PBS to produce a working solution. The fluorochrome solution and the whole blood were mixed in a 1:1 proportion. Immediately after, the samples were analysed with an epifluorescence microscope (Olympus BH-2, Kansas, USA) at 400X equipped with a 515–560 nm excitation filter and a barrier filter of 590 nm. Under these conditions, living cells can be identified for their green fluorescence and dead ones have a red color. Erythrocytes do not stain. In the present study, cell viability was obtained by counting the proportion of red and green cells. A sample of 200 cells was scored for each mouse. Samples for each animal were analysed in duplicate.

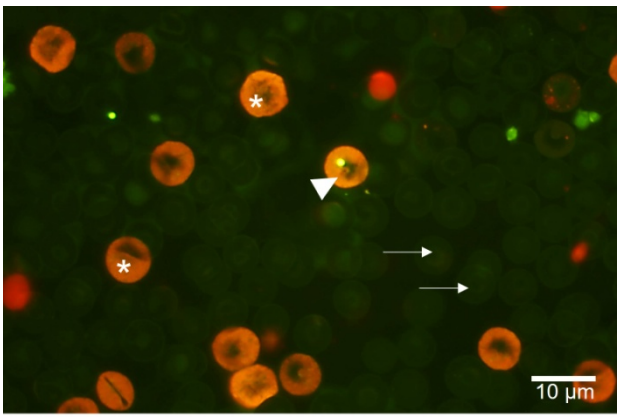

Figure 1. Micronucleus assay with slides covered with acridine orange and analyzed at 400X with an epifluorescence microscope. Mature erythrocytes (arrows), reticulocytes with micronuclei (arrowhead) and reticulocytes without MN (asterisks)

Peripheral blood micronucleus test *in vivo*: A micronucleus test was performed with some modifications, according to Hayashi et al. [19] and Rojas-Lemus et al. [20]. Briefly, we´ll describe the technique. Acridine orange (AO) fluorochrome was dissolved in distilled water (1 mg/mL). Then, 50 mL of the AO solution was placed on a previously cleaned and heated slide (~90°C). Afterwards, 4 µL of peripheral blood was collected from a cut of mice tail vein. It was then placed on an AO pre-covered slide protected with coverslips and maintained at 4°C for at least 24 h (to maximize dyeing). A duplicated sample was taken from each mouse. The percentage of polychromatic erythrocytes or peripheral blood reticulocytes (red-orange cells) was determined using 1,000 normochromic erythrocytes or mature erythrocytes (dark cells) and the micronucleus frequency was determined using 2,000 reticulocytes per animal, five animals per group. Micronuclei were easily recognized in the reticulocyte cytoplasm because of their strong green-yellowish fluorescence (Figure 1). Samples were analyzed at 400X with an epifluorescence microscope (Olympus BH-2, Kansas, USA) equipped with a 515–560 nm excitation filter and barrier filter of 590 nm.

Single Cell Gel Electrophoresis (SCGE): The alkaline SCGE or comet assay was performed as described by Rivera et al. [13]. Briefly, we´ll describe the technique. 5 µL of whole blood were obtained by a caudal vein cut of each mouse and mixed with 75 µL of 0.5% of low melting point agarose, 75 µL of this mixture was pipetted onto a slide previously layered with 150 µL of agarose and then covered with a coverglass (40 X 50 mm) to make a microgel on the slide. Slides were kept in an ice-cold tray to let the agarose turn into gel. The coverglass was gently removed, and 75 µL of agarose were layered as before. Slides were immersed in lysing solution (2.5 M NaCl, 100 mM Na_2EDTA, and 10 mM Tris-base, Triton X-100 [1%], DMSO [10%], pH 10, 4°C) for at least 24 h. After lysis, the slides were placed in a horizontal electrophoresis chamber. The DNA was allowed to unwind for 20 min in an electrophoresis running buffer solution (300 mM NaOH and 1 mM Na_2EDTA, pH ≥ 13). An electrophoresis was conducted for 20 min at 25 V and 300 mA. All the technical steps were conducted by using very dim indirect light. After the electrophoresis, the slides were removed and alkaline pH was neutralized with 0.4 M Tris, pH 7.5. The slides were dehydrated in three steps with absolute ethanol for 5 min each. Ethidium bromide (20 µL of a 20 mg/mL solution) was added to each slide and a coverglass was placed over the gel. DNA migration was analyzed on the Olympus BMX60 microscope with fluorescence equipment and measured with a scaled ocular as the tail length (in µm). For the evaluation of DNA migration, 200 cells were scored for each individual.

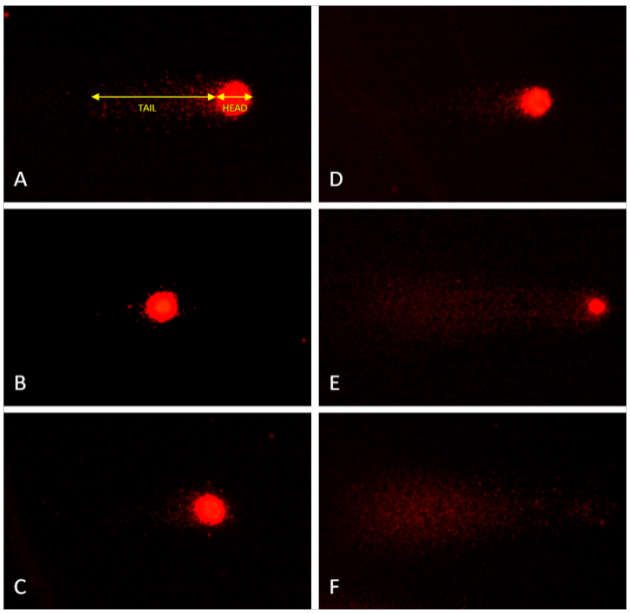

Figure 2. Comet assay DNA damage categorization. The sample is stained with ethidium bromide and analyzed at 400X with an epifluorescence microscope equipped with a scaled ocular. A) Measuring the length of the tail/head length (in µ), B) No damage, C) Low damage, D) Medium damage E) Severe damage and F) Total damage (cloud)

Next, the cells were classified into five categories according to Rodriguez-Mercado and coworkers [21]. The value of the nucleus ratio (r = measurement of the tail / measurement of the head) is as follows: (A) No DNA damage; (B) $r \leq 1$, Low DNA damage; (C) $1 < r \geq 2$, Medium DNA damage; (D) $r > 2$, Severe DNA damage; and (E) Total damage (Figure 2). Results were expressed as a "Migration Index" (MI), values were calculated according to the following formula: MI = [(A)1+(B)2+(C)3+ (D)4+(E)5] / total cells analyzed (%). A, B, C, D and E are the percentage of cells in each category and the numbers (1–5) are arbitrary units represented by each category. The MI considers all the damaged cells, including clouds (cells with total damage), which were excluded from the analysis in several cases.

2.3. Statistical Analysis

For the statistical analyses, GraphPad Prism® version 6.00 Software (GraphPad Software, San Diego, California, USA) was used. The D'Agostino and Pearson omnibus normality test was used to verify the normality of the data distribution of all the groups.

All values are presented as means ± standard error. One-way analysis of variance (ANOVA) and Tukey's Multiple Comparison post hoc test were used for the analysis of cell viability and micronucleus frequency. The equality of the variances was verified by the Bartlett´s test. Student t test (unpaired, two tailed) was employed in the comparison between the control and low-dose group. Pearson's correlation index was applied to analyze the association between doses and administration time. The study was performed with five mice per group. Cell samples were obtained from each group in duplicate and coded in a double-blind manner.

3. Results

No mice died during the experiment. Furthermore, no clinical signs of toxicity were observed.

Dual fluorochrome-mediated viability assay in peripheral blood leukocytes: The percentage of viable cells was determined each week during the administration of ascorbic acid. At time zero (T0), all groups had cell viability over than 92% during all experimental time.

In contrast, at 48 h, the cell viability of colchicine groups (88.67 ± 2.0) and MMS (85.67 ± 0.8) had a significant decrease ($p \leq 0.5$).

In general, in all the experimental groups cell viability was maintained above 80%, which is the minimum percentage required to perform the comet assay [15]. The raw data of acid ascorbic viability groups are presented in Table 1.

Peripheral blood micronucleus test in vivo: During all experimental time, the frequency of micronuclei (MN) in peripheral blood reticulocytes increased significantly in the 100, 150 and 225 mg/kg groups compared to time zero and the control group (0 mg/kg). Pearson's correlation index was positive and significant ($r^2 = 0.92$, $p=0.0254$) only for the highest dose group (225 mg/kg).

The 100 mg/kg group showed a significant decrease in the frequency of MN on the 3rd and 4th weeks compared with the 150 and 225 mg/kg groups.

Table 1. Percentage of viable cells in mice that were administered at different doses of ascorbic acid by oral route for 4 weeks. No differences were observed between groups. Data are shown in mean ± standard error

Time (weeks)	Dose (mg/kg/day)				
	0	50	100	150	225
0	96 ± 1.1	94.3 ± 1.3	96.5 ± 0.5	96.0 ± 1.0	95.0 ± 1.0
1	96 ± 1.3	96.8 ± 0.8	95.7 ± 1.7	95.7 ± 1.7	94.0 ± 1.0
2	95 ± 1.0	94.6 ± 2.3	93.7 ± 0.7	93.5 ± 1.5	94.0 ± 2.0
3	95 ± 0.1	94.4 ± 2.6	93.5 ± 1.5	94.2 ± 1.2	93.5 ± 0.5
4	95 ± 0.2	93.0 ±1.0	94.5 ± 0.5	94.0 ± 3.0	95.5 ± 0.5

The lowest dose (50mg/kg) group was the only one that did not show significant changes with respect to time zero or compared with the control group (0 mg/kg) (Figure 3).

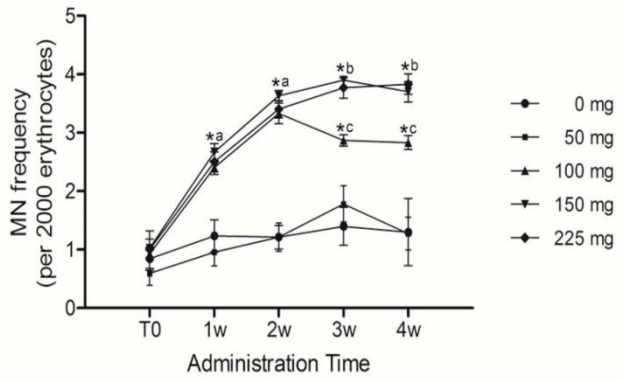

Figure 3. Micronuclei frequency in peripheral blood reticulocytes in male mice supplemented with oral ascorbic acid. The groups 100, 150 and 225 mg/kg/day showed a significant increase in the micronucleus count during all experimental time (4 weeks). No difference was observed in groups 0 and 50 mg/kg during all experimental time. ANOVA and Tukey's Multiple Comparison Test. *P≤0.05 (administration time vs T0), [a] $p \leq 0.05$ (100, 150 and 225 mg/kg *vs* 0 and 50 mg/kg), [b] $p \leq 0.05$ (150 and 225 mg/kg vs 100, 50 and 0 mg/kg), [c] $p \leq 0.05$ (100 mg/kg vs 50 and 0). Pearson's correlation index MN: 225 mg/kg $r^2 = 0.9230$.

As it was expected, in the positive-genotoxic control group the frequency of MN in reticulocytes was high, 7.8 ± 0.9 for MMS and 9.2 ± 2.1 for Col. These data are not shown in the graph.

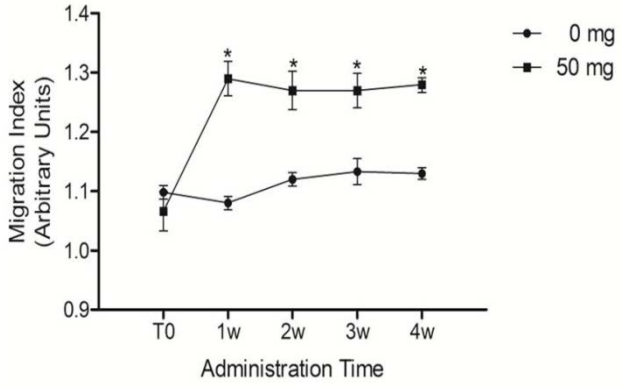

Figure 4. DNA Migration Index in peripheral blood leukocytes in mice that consumed a low dose of ascorbic acid orally. A significant increase from week 1 until the end of experimental time was observed. * $p \leq 0.05$ Student t test (50 mg/kg compared to 0 mg/kg)

Single Cell Gel Electrophoresis (SCGE) or Comet Assay: Only the group with the lowest dose and controls were included in this trial. The alkaline comet assay showed that the lowest dose (50 mg/kg) induced a significant increase in DNA single-strand breaks and therefore in the DNA migration index throughout the entire experimental time (Figure 4).

At 48 h post-administration, MMS induced a significant increase in the rate of leukocyte migration in the experimental mice (1.4 ± 0.15, $p \leq 0.05$), but not in the mice on the Col group (1.06 ± 0.022, $p \leq 0.05$). These data are not shown in the graph.

4. Discussion

Ascorbic acid is not a cytotoxic agent

Our results show that ascorbic acid at different doses did not cause cytotoxic effects on leukocytes, and we did not observe a decrease on the percentage of living cells in the experimental mice.

This result indicates that the cells from experimental animals, despite the damage caused to the genetic material, remain alive and, therefore have the potential to become aberrant cells.

Genotoxic effects of acid ascorbic: Micronuclei and DNA strand breaks.

Our results indicate that even low doses of ascorbic acid orally administered cause genotoxic effects. We use "low doses" because the reported range in experimental studies of this antioxidant goes from 5 mg to 2,500 mg per day [22]. In contrast with our results, in a study with healthy humans that consumed different doses of ascorbic acid (80, 200 and 400 mg/day) an increase of oxidative damage to the DNA was not observed [23].

In cancer cell lines, *in vitro* studies showed that ascorbic acid generates pro-oxidant effects, inhibition of cell growth, cell death by apoptosis or necrosis, DNA damage (strand breaks), mitochondrial damage, decreases cell survival and cell cycle disruption. In contrast, other studies with these same cell lines report that it stimulates growth and does not cause adverse effects. Du et al. present a detailed review about this [5].

In the above studies, it is important to emphasize that the pro-oxidant effects of ascorbic acid were described in aberrant cell lineages, whose physiology is different from normal cells of healthy individuals. In studies of cancer patients, increasing evidence suggests that vitamin C has the potential to be a potent anti-cancer agent when administered intravenously and in high doses, as clinical trials have indicated its efficacy in eradicating tumor cells in several types of cancer [24]. However, although vitamin C is relatively safe, caution should be exercised when administering high doses, which may cause overt side effects in some susceptible patients [25].

Moreover, the main *in vivo* model is the nude mouse. This experimental model has helped to test, in various types of tumours, the effects of ascorbic acid. The results show that the administration of an antioxidant by different routes is usually beneficial, because in most cases, a decrease in the size of the tumour [5] is reported. However, this mouse model does not represent healthy mice, which were the type of mice used in this work.

Only a few experimental studies have suggested that ascorbic acid has antioxidant and pro-oxidant activity on *in vivo* systems.

Our results indicate that the ascorbic acid in healthy mice produces adverse effects on the DNA, identified as single-strand breaks and micronuclei. This DNA damage could be caused by the pro-oxidant effects, which have been attributed to ascorbic acid. DNA damage caused by oxidative stress comprises oxidation and modification of the nucleotide's components (nitrogenous bases and sugar deoxyribose), crosslinks and strand breaks [26,27], all of them are damages that may generate micronucleus in proliferating cells. Any agent that reacts with DNA is potentially dangerous [28]; DNA damage can lead to mitotic arrest (among other alterations in cell cycle progression), induction of gene transcription and replication errors in genomic instability; all of which are involved in carcinogenic processes [24].

In future research, it will be important to identify the etiology of micronuclei (clastogenic or aneugenic origin) caused by ascorbic acid. This could help us understand more about the action mechanism that makes this important antioxidant also a genotoxic agent.

5. Conclusion

In conclusion, the consumption of ascorbic acid when there it is no associated pathologies can be dangerous because this vitamin has genotoxic potential *per se* and its indiscriminate use may cause more damage than benefit.

This experimental work shows the effect of oral administration of ascorbic acid in healthy mice on the DNA. No genotoxic effects have been reported in similar studies because in the existing, ascorbic acid is administered only when the experimental subjects present important health issues, which completely changes the focus of the results observed. The genotoxic effects that we observe in these experimental conditions are small. However, it should be considered that the administration lasted only 4 weeks and that the doses were low. But in daily life, situations can be very different because anyone can access and consume higher doses of this vitamin and for a longer period. Hence, the results presented are very valuable.

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