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# Genetic damage induced by $CrO_3$ can be reduced by low doses of Protoporphyrin-IX in somatic cells of *Drosophila melanogaster*

Luz M. Vidal E.[a], Emilio Pimentel P.[a,*], M. Patricia Cruces M.[a], Juan C. Sánchez M.[b]

[a] *Departamento de Biología, Instituto Nacional de Investigaciones Nucleares (ININ), Carretera México-Toluca, S/N, la Marquesa, Ocoyoacac, CP. 52750, México*
[b] *Facultad de Química, Universidad Autónoma del Estado de México, Toluca, México*

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ABSTRACT

Several epidemiological studies have reported the relation between chromium exposure (used in different industrial processes) and cancer risk. Evidence indicates that the hexavalent form is mutagenic and carcinogenic. Chemoprevention has emerged as a good strategy for reducing the risk from exposure to heavy metals. There is evidence that some tetrapyrrols such as protoporphyrin IX (PP-IX), a porphyrin without a metal center and which is a precursor of hemoglobin and cytochrome, acts as an antioxidant modulating the induction of antioxidant enzymes. The present study was performed to evaluate their antimutagenic potential of PP-IX against genetic damage induced by chromium trioxide ($CrO_3$). The wing spot test was used. Groups of 48 h-old larvae were pretreated for 24 h with 0, 0.69, 6.9, or 69 mM of PP-IX, after which groups of larvae were fed 0.025–2.5 mM $CrO_3$ solution in *Drosophila* instant medium. The results indicated that the lower PP-IX concentration (0.69 mM) significantly reduced the genetic damage induced by all $CrO_3$ concentrations tested. In contrast, 6.9 and 69 mM only inhibited the damage induced by $CrO_3$ 2.5 mM. Absence of an inhibitor effect of PP-IX against 20 Gy gamma rays suggested that this porphyrin acted primarily by forming complexes with chromium at low doses, inactivating its genotoxic action rather than capturing or inactivating the reactive oxygen species generated by the chromium.


## 1. Introduction

Exposure to environmental pollutants of anthropogenic origin is associated with an important increase of chronic degenerative diseases including cancer [1]. Although heavy metals are present naturally in soils, their contamination is caused directly by industrial and mining activities. Metals such as Pb, Hg, Cd, As, Ce, and Cr are very harmful to human health and to most living organisms. They are not chemically or biologically degradable components which accumulate in the soil. If additionally the metals are filtered in groundwater, control is very difficult and the metals can enter the food chain, either through drinking water or through consuming contaminated crops in agricultural soils, becoming a potential health risk [2]. They have been associated with diseases such as pneumonia, renal dysfunction, emphysema, and bone cancer [3], as well as with an impaired nerve function system [4].

*Abbreviations:* PP-IX, protoporphyrin IX; ROS, reactive oxygen species; SMART, Somatic Mutation and Recombination Test; CAT, catalase; SOD, superoxide dismutase; ENU, N-nitroso-N-ethylurea.

* Corresponding author. Tel.: +55 5329 7200.
*E-mail address:* emilio.pimentel@inin.gob.mx (E. Pimentel P.).

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Given this situation, the need arises to implement strategies that will reduce the occurrence of degenerative diseases. The first, and the most obvious, is to avoid exposure of human beings to those agents that have the ability to modify the genetic material and, therefore, increase the risk of diseases such as cancer. However, in practice this is almost impossible since many of such agents are found naturally in the atmosphere (as ultraviolet or ionizing radiation), and others are products of the metabolism of innocuous compounds such as nitrates [5]. The second alternative is to increase the consumption of substances capable of preventing or reducing the adverse effects of mutagenic and carcinogenic agents. This strategy refers to chemoprevention and it is defined as the use of chemical compounds, especially those of natural origin that cause inhibition or reversal of mutagenesis or carcinogenesis in the premalignant state [1].

Among heavy metals, chromium is considered one of the most dangerous and the International Agency of Research on Cancer [6] recognized the carcinogenicity of this metal. One of the biggest sources of Cr VI exposure is through the release of particles during stainless steel solder [7,8]. Exposure pathways include the following: oral, respiratory, and dermal pathways [9]. Chromium has been reported to be distributed into the body through the bone marrow, lungs, lymph nodes, spleen, kidney, and liver [10,11]. The danger of this element is known to occur during the reduction process of Cr (VI) to Cr (III), generating reactive oxygen species (ROS) that interact with the genetic material and are able to induce various alterations, for example: sister chromatid exchanges, chromosomal aberrations and cross-links in double-stranded DNA [11]. Not only are workers in manufacturing industries exposed to chromium compounds, but also the general public is exposed through cigarette smoke, automobile emissions, landfills, and hazardous waste disposal sites [12].

Among the natural compounds with chemoprevention properties, porphyrins are aromatic heterocyclic macrocycles derived from the porphine base structure. They are considered promising mainly because of their low toxicity. These molecules have the ability to form complexes directly with planar polycyclic structures thereby preventing their interaction with DNA; however, tetrapyrroles can also be interspersed in the molecule [10]. The antimutagenic activity of porphyrins containing some metal ion is due to their antioxidant effect [13–15]. Protoporphyrin-IX is a molecule with no metal core, it comes from the biosynthesis of 5-aminolevulinic acid and it is the immediate precursor of heme [16]. The toxic effect of PP-IX could probably be due to the imbalance of the redox systems in the over production of ROS [17,18], which leads to an increase in lipid peroxidation, which in turn is the main cause of damage to the liver [19,20]. In rats of the CF1 strain, PP-IX induced lipid peroxidation and increased the activity of catalase (CAT) 30% after 2 h of administration while the superoxide dismutase (SOD) enzyme increased 24 h after administration, yet peroxidation decreased after several doses of PP-IX [21]. Chronic treatment with PP-IX was recently found to prolong the lifespan of a wild *Drosophila* strain. In contrast, half life was reduced in the Sod deficient strain; these data provide information that PP-IX can act as an antioxidant and as a pro-oxidant [22]. Based on these previous works, the aim of the present study was to evaluate the antimutagenic capacity of PP-IX against the genetic damage induced by $CrO_3$ (VI), an agent that induces genetic damage especially through ROS.

## 2. Materials and methods

*Somatic Mutation and Recombination Test (SMART)*: In order to test DNA damage, we performed the wing spot test [23] as follows: three-day-old *mwh +*/*mwh +* females and *flr*$^3$/In (3LR), TM3, *Bds* males were mated for 2 h, then transferred to an egg-laying surface. Oviposition was restricted to a 2 h period so as to obtain more homogeneous samples in the age of individuals under test. Then 48 h-old larvae were collected by density gradient using a 20% sucrose solution. They were subsequently washed with $24 \pm 1\,^{\circ}C$ tap water and pretreated for 24 h in the dark, in flasks (1/4 L) with a paper filter (Whatman # 2), saturated with PP-IX 0, 0.69, 6.9, or 69 mM dissolved in a 5% sucrose solution. Distilled water was used for all solutions.

On completion of the pretreatment period, the larvae were washed with tap water at $24 \pm 1\,^{\circ}C$. At this time, aliquots of larvae from each pretreatment concentration of PP-IX were treated with 0.025, 0.25, 1.25, 2.0, or 2.5 mM $CrO_3$ in plastic vials (9.5 cm height and 2.5 cm diameter) with 1.5 g of *Drosophila* instant medium (formula 4-24 Carolina Biol. Supply Co.). Experiments were carried out in triplicate for each pretreated PP-IX solution and for each treatment with $CrO_3$ solution. All treatments were conducted in the dark.

Upon eclosion, organisms were fixed in 70% alcohol and the wings of the *mwh +*/*+ flr*$^3$ flies (i.e., non-Ser) were mounted on slides for 40× microscopic analysis. The wings were examined to identify small single spots (one or two cells), large single spots (more than two cells) of either *mwh* or *flr*, and *mwh-flr* twin spots. Single *mwh* spots are expected to be produced following mutation at the *mwh +* locus or by an interchange between the *mwh* and *flr*$^3$ loci succeeded by homozygosis for *mwh*. Single *flr*$^3$ spots may arise by mutation at the *flr*$^{3+}$ locus or by double exchange. Twin spots are the result of an interchange between the *flr*$^3$ and the centromere [23]. For a description of the mutants see Lindsley and Zimm [24].

All data for each group represent at least two experiments performed in triplicate. Results from the groups treated with PP-IX and $CrO_3$ were compared with the group corresponding to each $CrO_3$ concentration. The SMART statistical program proposed by Frei and Würgler [25] was used to determine differences between treatments. The criterion for significance was set at $p < 0.05$.

*Toxicity test:* In order to quantify the toxicity provoked by PP-IX and $CrO_3$ by themselves, groups of fifty 48h-old larvae *mwh +*/*+ flr*$^3$ were collected and treated following the method described above using the same concentrations of PP-IX and $CrO_3$. To evaluate toxicity of the combined treatment of the different PP-IX concentrations, we selected the highest $CrO_3$ concentration (2.5 mM). Chi square test was used for statistical analysis at $p < 0.05$ level. PP-IX and $CrO_3$ were purchased from Sigma Chemicals Co. (St. Louis, MO).

**Table 1**
Spots frequency induced in 48 h-larvae *mwh +/ + flr³* by different concentrations of chromium trioxide (VI).

| Treatment (mM) | | Spots per wing (number of spots) | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Small single spots | | Large single spots | | Twin spots | | Total spots | |
| $CrO_3$ | No. of wings | (1–2 cells), $m=2$ | | (>cells), $m=5$ | | $m=5$ | | $m=2$ | |
| 0 | 160 | 0.24 | (39) | 0.09 | (14) | 0.01 | (1) | 0.34 | (54) |
| 0.025 | 120 | 0.26 | (31) – | 0.04 | (5) – | 0.03 | (4) i | 0.33 | (40) – |
| 0.25 | 150 | 0.43 | (64) + | 0.11 | (16) – | 0.05 | (8) + | 0.59 | (88) + |
| 1.25 | 118 | 0.63 | (74) + | 0.36 | (42) + | 0.14 | (16)+ | 1.12 | (132)+ |
| 2.0 | 80 | 0.81 | (65) + | 0.52 | (42) + | 0.45 | (36)+ | 1.79 | (143)+ |
| 2.5 | 120 | 2.87 | (344) + | 1.97 | (236) + | 2.11 | (254)+ | 6.95 | (834)+ |

Statistical diagnoses according to Frei and Würgler [25]: + = positive; – = negative; w = weak positive; i = inconclusive; $m$ = multiplication factor. Probability levels: alpha = beta = 0.05. One-side statistical test.

*Treatment with gamma rays:* To explore activity of the PP-IX as a radical scavenger, we collected 48 h-old *mwh +/+ flr³* larvae and pretreated them for 24 h with PP-IX following the method described above. On completion of the pretreatment, larvae from each concentration were divided into two groups, one of each group was irradiated with 20 Gy gamma rays in a Transelektro LGI-01, Co-60 irradiator with a dose rate of up to 1259.44 Gy/h; the other larva served as control. After irradiation, larvae were put into a plastic vial with hydrated medium formula 4-24. All the vials were introduced into a culture room until the development concluded. The wings analysis was done as described earlier.

## 3. Results

Table 1 shows the frequency of all kinds of spots induced by the different concentrations of $CrO_3$. Statistical significant differences were found for all kinds of spots from 0.25 mM and higher concentrations. Noticeably, the higher concentration provoked a frequency of mutation that represents 20 times the frequency found in the control group.

Table 2 includes the results obtained with the pretreatment of 0.69, 6.9, or 69 mM of PP-IX and with the combined treatment of PP-IX + $CrO_3$: Sections 1, 2, and 3, respectively. Comparison of the action of PP-IX alone indicated that only 69 mM doubled the basal mutation frequency.

The statistical analysis comparing the combined treatments with their respective positive control in Table 1 indicated that pretreatment with 0.69 mM of PP-IX caused a reduction in the frequency of all kinds of spots from PP-IX + 0.025 mM $CrO_3$, however, this reduction were not statistically significant for all kinds of spots. A statistically significant reduction was found from PP-IX 0.69 + $CrO_3$ 0.25 mM, which decreased the twin spots induced by all concentrations of $CrO_3$ with the exception of the PP-IX + 1.25 mM group. Pretreatment with PP-IX 6.9 mM caused only a slight reduction in the frequency of the

**Table 2**
Spots frequency induced by chromium trioxide (VI) treatment in 48 h-larvae *mwh+/+flr³* pretreated 24 h with 0.69, 6.9 or 69 mM of PP-IX.

| Treatment (mM) | | Spots per wing (number of spots) | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Small single spots | | Large single spots | | Twin spots | | Total spots | |
| PP-IX (+) $CrO_3$ | No. of wings | (1-2 cells), $m=2$ | | (>cells), $m=5$ | | $m=5$ | | $m=2$ | |
| | Pretreatment with PP-IX 0.69 mM (Section 1) | | | | | | | | |
| 0.69 | 120 | 0.33 | (40) i | 0.05 | (6) – | 0.03 | (3) i | 0.41 | (49) – |
| +0.025 | 80 | 0.16 | (13)i | 0.02i | (2) i | 0.02 | (2) i | 0.21 | (17) i |
| +0.25 | 80 | 0.30 | (24)i | 0.06 | (5) – | 0.01 | (1) i | 0.37 | (30)+ |
| +1.25 | 80 | 0.41 | (80)+ | 0.10 | (8) + | 0.14 | (11) – | 0.65 | (52)+ |
| + 2.0 | 80 | 0.70 | (56) – | 0.32 | (26) – | 0.26 | (21)+ | 1.30 | (103)+ |
| + 2.5 | 120 | 0.57 | (69)+ | 0.32 | (38) + | 0.28 | (33)+ | 1.17 | (140)+ |
| | Pretreatment with PP-IX 6.9 mM (Section 2) | | | | | | | | |
| 6.9 | 120 | 0.23 | (28) – | 0.05 | (6) – | 0 | (0) | 0.28 | (34) – |
| + 0.025 | 40 | 0.26 | (8) i | 0.08 – | (3) – | 0 | (0) | 0.28 | (11) i |
| + 0.25 | 80 | 0.31 | (25) i | 0.14 | (11) – | 0.04 | (3) i | 0.49 | (39) – |
| + 1.25 | 80 | 0.51 | (41) – | 0.37 | (30) – | 0.12 | (9) – | 1.00 | (80) – |
| + 2.0 | 80 | 0.59 | (47) + | 0.31 | (25) – | 0.30 | (24) – | 1.20 | (96)w |
| + 2.5 | 120 | 0.64 | (77) + | 0.32 | (38) + | 0.23 | (28)+ | 1.19 | (143) + |
| | Pretreatment with PP-IX 69 mM (Section 3) | | | | | | | | |
| 69 | 160 | 0.51 | (81) + | 0.14 | (23) – | 0.01 | (2) i | 0.66 | (106) + |
| + 0.025 | 120 | 0.37 | (45) – | 0.08 | (10) – | 0 | (0) | 0.46 | (55) – |
| + 0.25 | 120 | 0.32 | (39) – | 0.08 | (9) – | 0.02 | (2) i | 0.42 | (50) i |
| + 1.25 | 80 | 1.51 | (121) – | 0.56 | (45) – | 0.40 | (32) – | 2.47 | (198) – |
| + 2.0 | 80 | 1.10 | (87) – | 0.60 | (48) – | 0.54 | (43) – | 2.22 | (178) – |
| + 2.5 | 140 | 0.92 | (129) + | 0.46 | (64) + | 0.40 | (56)+ | 1.78 | (249) + |

Statistical diagnoses according to Frei and Würgler [25]: + = positive; – = negative; w = weak positive; i = inconclusive; $m$ = multiplication factor. Probability levels: alpha = beta = 0.05. One-side statistical test. The comparisons were between each group: PP-IX + $CrO_3$ concentration with the respective positive control included in Table 1. The signs indicated in the PP-IX concentration alone are the results from comparisons with the negative control.

**Table 3**
Toxicity of 48 h-old *mwh+/+flr³* larvae after the treatment for 24 h with PP-IX, $CrO_3$ or combined treatment.

| Treatment (mM) | No. of larvae | No. of viable larvae ± *SEM* | % of non-viable larvae |
|---|---|---|---|
| 0 | 500 | 420 ± 0.9 | 16 |
| PP-IX | | | |
| 0.69 | 500 | 394 ± 1.7 | 21 |
| 6.9 | 500 | 447 ± 0.6 | 11 |
| 69 | 500 | 368 ± 0.5 | 26 |
| $CrO_3$ | | | |
| 0.25 | 500 | 448 ± 0.3 | 10 |
| 2.0 | 500 | 325 ± 2.8 | 35* |
| 2.5 | 500 | 194 ± 0.8 | 61* |
| PP-IX + $CrO_3$ | | | |
| 0.69 + 2.5 | 400 | 164 ± 1.2 | 59 |
| 6.9 + 2.5 | 500 | 216 ± 2.0 | 57 |
| 69 + 2.5 | 500 | 230 ± 1.8 | 54** |

* Significant to $p < 0.05$. Compared with control
** Significant to $p < 0.05$. Compared with $CrO_3$ 2.5 mM

different kinds of spots from the PP-IX + 0.25 mM group, and only a weak decrease with respect to damage caused by 2.0 mM $CrO_3$; however, this concentration of PP-IX plus 2.5 mM $CrO_3$ fell six times the frequency of total spots induced by the mutagen alone (2.5 mM). Comparison of the effect of pretreatment with the 69 mM concentration and the previous two concentrations indicated that the combined treatment PP-IX + $CrO_3$ 2.5 mM group decreased four times the frequency of total spots induced by $CrO_3$ alone.

Results of toxicity for different concentrations of PP-IX, $CrO_3$ (0.25, 2, and 2.5 mM) combined treatments are presented in Table 3. The analysis showed that PP-IX was nontoxic at the three concentrations tested compared to the control. $CrO_3$ was toxic at 2 and 2.5 mM; the latter reduced viability 45% over the control. In contrast the combined treatments reduced toxicity, but 69 PP-IX + 2.5 $CrO_3$ increased viability 7% compared with the 2.5 mM $CrO_3$ control group. Table 4 presents the results obtained for somatic mutation when larvae were pretreated with different concentrations of PP-IX and then treated with 20 Gy of gamma rays. The results indicated that PP-IX in all tested concentrations had no effect on damage induced by gamma rays.

## 4. Discussion

Cr [VI] is known to produce breaking in one or both strands of DNA; DNA-DNA bonds; DNA cross-links with proteins, besides modifying the nucleotides as is the case of guanine, 8-hydroxyguanine links. Although the mechanisms involved are not yet very clear, all DNA alterations can cause chronic degenerative diseases, including cancer [26].

Genetic damage induced by chromium is mainly produced as a result of its ability to induce free radicals, especially during the reduction from Cr [VI] to Cr [III]. Such radicals are the superoxide anion ($O^{2-}$), and hydroxyl radical (OH•) [27]. To counteract the action of ROS, cells have a system of endogenous enzymes such as SOD and CAT and the glutathione as antioxidants. Furthermore, exogenous antioxidants such as vitamins may reduce the cellular damage caused by free radicals. The main subject of the present study was to evaluate the antioxidant capacity of PP-IX avoiding the genetic damage induced by $CrO_3$. There is evidence that the antioxidant action of this tetrapyrrol depends on its concentration [21,28,29]. The results obtained in our study provided evidence of this effect: PP-IX was not toxic by itself (Table 3) and concentrations of 0.69 and 6.9 mM did not induce genetic damage, yet the 6.9 mM reduced total spots ($p < 0.05$) compared to control and also reduced toxicity 5% but not significantly (Table 3) suggesting that protoporphyrin can act as a true antimutagen. Moreover, the highest concentration (69 mM) proved to be mutagenic and induced damage comparable with 0.25 mM of $CrO_3$. In agreement with this, PP-IX has been proven to induce oxidative stress [21] via production of superoxide ions and more efficiently of singlet oxygen in organic solutions [30]. Our results are in accord with this finding. Pimentel et al. [31] found that this pigment at 69 mM can be mutagenic 24 h after its administration in combination with 0.5 mM N-nitroso-N-ethylurea (ENU) and that such activity may persist for 72 h. However, the evidence found in this study indicated that in organisms pretreated with PP-IX and subsequently treated with $CrO_3$, the frequency of genetic damage was reduced in most of the treated groups.

Worth noting is the effect of the lowest concentration of PP-IX, (0.69 mM) which caused a significant decrease of damage in four of the five concentrations of $CrO_3$ tested. In comparison, 6.9 mM significantly decreased the frequency of damage induced by 2 and 2.5 mM of the mutagen, and the highest concentration (69 mM) only reduced the damage induced by 2.5 mM. Even so, 2.5 mM of $CrO_3$ provoked high mortality (61%) which could result in loss of information;

**Table 4**
Spots frequency induced in 48 h-old *mwh +/+ flr³* larvae pretreated 24 h with different concentrations of PP-IX and subsequently treated with 20 Gy of gamma rays.

| Treatment | | Spots per wing (number of spots) | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| PP-IX (mM) + 20 Gy | No. of | Small single spots | | Large single spots | | Twin spots | | Total spots | |
| | wings | (1-2 cells), *m* = 2 | | (>cells), *m* = 5 | | *m* = 5 | | *m* = 2 | |
| 0 | 40 | 0.27 | (11) | 0.05 | (2) | 0.02 | (1) | 0.35 | (14) |
| 20 Gy | 80 | 0.70 | (56) | 1.57 | (126) | 0.06 | (5) | 2.33 | (187) |
| 0.69 + | 80 | 0.82 | (66) – | 1.16 | (93) w | 0.08 | (7) – | 2.07 | (166) – |
| 6.9 + | 80 | 0.69 | (55) – | 1.84 | (147) - | 0.15 | (12) – | 2.67 | (214) – |
| 69 + | 80 | 1.16 | (93) – | 1.52 | (122) – | 0.07 | (6) – | 2.76 | (221) – |

Statistical diagnoses according to Frei and Wüergler [25]: + = positive; – = negative; w = weak positive; i = inconclusive; *m* = multiplication factor. Probability levels: alpha = beta = 0.05. One-side statistical test.

in combination with PP-IX, 69 mM increased viability by 7% and provoked a net effect of reducing 3 times inhibition of the genetic damage induced by $CrO_3$ 2.5 mM ($p < 0.05$).

Explaining the increase in mutation frequency obtained in the groups 69 + 1.25 and 2 mM $CrO_3$ is not simple. However, this increase could suggest a synergistic effect of $CrO_3$ plus the pro-oxidant action of PP-IX for its accumulation generating superoxide, as suggested by Afonso et al. [21]. PP-IX reacts with molecular oxygen-producing peroxide radicals that cause lipid peroxidation and lead to different cell damage such as structural changes in the cell membrane, damage to proteins, inactivation of receptors, enzymes, and ion channels, all of which can lead to cell death [32]. Other studies have demonstrated that porphyrins can bind to DNA via a specific insertion within only one strand of DNA, i.e., by hemi-intercalation [33] with a binding constant of around 106 $M^{-1}$ [34,35]. In this way, these extra-helical structural elements could be a factor in certain pathways of mutagenesis [21].

The fact that the lowest concentration of PP-IX provoked a significant decrease in damage could be due to the fact that the porphyrin ring could make complexes or act as a chelator, introducing the chromium into the ring. PP-IX has been reported to be able to bind other metals such as zinc and nickel [36]. The different studies on the role of ferrochelatase have revealed that this enzyme catalyzes zinc as well as the iron chelating activity of protoporphyrin [14]. Ferrochelatase is known to catalyze insertion of divalent transition metal ions other than iron *in vitro*, most notably zinc, but cobalt, nickel, and copper have also each been reported to act as substrates, although species-specific differences have been noted [37–39]. Ferrochelatase could participate in the chelating chromium, provoking a reduction in its mutagenic effect. The work performed by Pimentel et al. [40] and Gaivão et al. [41] have demonstrated that the somatic mutation and recombination test in *Drosophila* is a useful tool that detects the very low doses of alpha particles and the ROS induced by oxidants agents, respectively; a possibility to explain our results is the ability of PP-IX to release electrons [42], which may have inactivated the free radicals generated by chromium thereby avoiding DNA damage. The studies performed by Afonso et al. [21] provide evidence of the action of PP-IX in generating superoxide and hydrogen peroxide, which causes activation of SOD and CAT enzymes that help to inactivate ROS produced by the chromium oxide-reduction reactions. In contrast, Cruces et al. [43] found that a pretreatment with very low doses of sodium copper chlorophyllin increased the somatic mutation frequency induced by 10 Gy gamma rays.

Results from the present work showed evidence indicating that the lower concentration of PP-IX reduced genetic damage of the radiomimetic agent $CrO_3$. To evaluate the ability of PP-IX as a radical scavenger, we tested its action against the effect of 20 Gy of gamma rays (Table 4). The comparison of the effect of PP-IX against the two agents revealed that the action of PP-IX is more likely to be preferable through the formation of chemical complexes with chromium rather than through decreasing reactive oxygen species. These findings placed PP-IX as an effective antimutagen at low doses.

## Transparency document

The Transparency document associated with this article can be found in the online version.

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