Environmental Toxicology and Pharmacology 80 (2020) 103464

Contents lists available at ScienceDirect

Environmental Toxicology and Pharmacology

journal homepage: www.elsevier.com/locate/etap

Radioprotective effect of chloropyllin, protoporphyrin-IX and bilirubin compared with amifostine® in *Drosophila melanogaster*

E. Jiménez[a], E. Pimentel[a,*], M.P. Cruces[a], A. Amaya-Chávez[b]

[a] Instituto Nacional de Investigaciones Nucleares, Carretera México-Toluca s/n, La Marquesa, Ocoyoacac, Edo. de México, C.P. 52750, Mexico
[b] Facultad de Química, Universidad Autónoma del Estado de México, Toluca, Mexico

ARTICLE INFO

Keywords:
D. melanogaster
Somatic mutation
Porphyrins
Bilirubin
Radio-protector effect

ABSTRACT

The identification of substances that prevent or minimize the detrimental effects of ionizing radiation is an essential undertaking. The aim of this paper was to evaluate and compare the radioprotective potential of chlorophyllin, protoporphyrin and bilirubin, with amifostine®, an US Food & Drug Administration approved radioprotector Using the somatic mutation and recombination assay in the *Drosophila melanogaster* wing, it was found that pretreatment (1 − 9 h) with any of the porphyrins or amifostine® alone, did not affect the larva-adult viability or the basal frequency of mutation. However, they were associated with significant reductions in frequency of somatic mutation and recombination compared with the gamma-irradiated (20 Gy) control as follows: bilirubin (69.3 %) > chlorophyllin (40.0 %) > protoporphyrin (39.0 %) > amifostine® (19.7 %). Bilirubin also caused a 16 % increase in larva-adult viability with 3 h of pretreatment respect to percentage induced in 20 Gy control group. Whilst amifostine® was associated with lower genetic damage after pre-treatment of 1 and 3 h, this did not attain significance. These findings suggest that the tested porphyrins may have some potential as radioprotectant agents.

1. Introduction

The increased use of ionizing radiation (IR) in medical diagnosis, treatment, and the generation of energy together with the possibility of accidents or nuclear terrorism increases human exposition with the consequent health risks. For this reason, it is necessary to look for alternatives to avoid or minimize the adverse effects of IR (Kamran et al., 2016). Research in identification of radioprotectors is a strategy that is receiving attention for its health benefits, a radioprotective agent in principle should not be toxic and must have the ability to reduce the deleterious effects of IR (Venkatachalam and Chattopadhyay, 2005), it is also necessary that the hypothetical radioprotective agent be present in the cell medium before or during radiation exposure (Shirazi et al., 2007) because the free radicals (FR) have a very short life. The mechanisms of action of radioprotectors included: 1) Suppressing the reactive oxygen species formation (ROS); 2) Stabilizing ROS by donating electrons; and 3) Improving the repair cell mechanisms (Shirazi et al., 2007). A large number of compounds have been proposed as radioprotectors, because they have been shown to be efficient in *in vitro* systems, however most of them failed in *in vivo* tests due to their acute toxicity and unfavorable side effects (Weiss and Landauer, 2003).

Amifostine® (AMF®) [S-2- (3-aminopropylamino) etiol fosphorothioic acid] also known as WR 2721, is the only thiol that has been clinically approved as a radioprotector for use in humans by the FDA, to mitigate adverse effects in patients undergoing chemo and / or radiotherapy (Joshi et al., 2010). AMF® is a broad-spectrum cytoprotective compound and a selective agent to protect normal tissues from the cytotoxic effects of some drugs used in cancer therapy. Its selective effect for non-cancer cells is due to low alkaline phosphatase concentration in cancer cells than normal (Sasse et al., 2006 and Kouvaris et al., 2007). AMF® is a prodrug that has no activity until WR-1065 is dephosphorylated to its active metabolite by alkaline phosphatase enzyme in the plasma membrane (Andreassen et al., 2003), after activation, AMF® accumulates in cells and inactivate free radicals by donating H + from sulfhydryl groups in their structure, it also induces cellular anoxia, which prevents cellular damage caused by oxygen (Kouvaris et al., 2007). However, some preclinical studies indicated that it could protect not only normal cells but cancer cells as well, and produces side effects after administration such as: hypotension, nausea, and allergic reactions (Andreassen et al., 2003).

One alternative to avoid these side effects, is the identification of the radioprotective potential of naturally occurring compounds, which expected to be nontoxic (Venkatachalam and Chattopadhyay, 2005 and Kamran et al., 2016). Porphyrins are a group of widely distributed

* Corresponding author.
E-mail address: emilio.pimentel@inin.gob.mx (E. Pimentel).

https://doi.org/10.1016/j.etap.2020.103464
Received 26 April 2020; Received in revised form 29 July 2020; Accepted 30 July 2020
Available online 01 August 2020

macrocyclic compounds in nature that play a very important role in many metabolic pathways. The basic structure is the porphyrinic ring, composed by four pyrrole rings linked by methyl bridges and constitutes the prosthetic group of many molecules, such as hemoproteins, chlorophylls, cytochromes, etc. (Biesaga et al., 2000). There is experimental evidence indicating that some porphyrins such as protoporphyrin IX (PPIX); chlorophyll (CL); copper-sodium chlorophyllin (SCC); bilirubin (BRB) and biliverdin (BVB) have antioxidant and antimutagenic activity (Zimmering et al., 1990; Odin, 1997; Mölzer et al., 2012).

Protoporphyrin IX is part of the biosynthesis of the heme group and of most of the cytochromes and chlorophylls, it derives from 5-aminolevulinic acid (ALA), the second last compound of the synthesis (Greer et al., 2018). Both compounds, PPIX and ALA are used for medical purposes in photodynamic therapy besides chemo and radiotherapy. Structurally, PPIX is a plane tetrapyrrole lacking transition metal in the center, despite some experimental evidence has been obtained, the antioxidant properties of PPIX are not fully characterized (Mölzer et al., 2012). It has been shown its antioxidant activity in the dark, by inactivating peroxyl radicals and inhibiting the activity of the cytochrome P-450 system in rat liver microsomes (Williams et al., 1992, 1994). In an *in vivo* study, using somatic cells of *D. melanogaster*, it was found that PPIX has antimutagenic activity against chromium trioxide (CrO_3) (Vidal et al., 2014), and it also increases the longevity of this organisms, this finding suggests that PPIX provides protection against oxidative stress, avoiding the action of the superoxide radical (Pimentel et al., 2013).

Chlorophyllin is a semi-synthetic mixture of copper and sodium salts derived from chlorophyll; for its synthesis, the magnesium atom in the center of the ring and the phytol esters of CL are replaced by copper and sodium, respectively. As a result of these changes, SCC is more stable and soluble than CL (Sarkar et al., 1994, 1995). Given the structural similarity of the SCC with the prosthetic group of hemoglobin, some of the first uses of SCC were in patients with anemia and to decrease the coagulation time in bleeding. Currently SCC has been extensively studied and has been found to possess antimutagenic, anticarcinogenic, anticlastogenic and antioxidant properties against a wide variety of compounds in different systems (Tumolo and Lanfer-Marquez, 2012). Zimmering et al. (1990) reported the first evidence that SCC acts as radioprotector in somatic cells of *D. melanogaster* and found that a 69 mM SCC pretreatment significantly reduced the frequency of mutations induced by 20 Gy of gamma rays. Pimentel et al. (1999), demonstrated that this radioprotective effect persisted for up to 72 h after the end of pretreatment.

Bilirubin derives from the catabolism of the heme group in mammals and for many years it was considered a potentially cytotoxic waste product (Tomaro and del C Batlle, 2002; Mancuso, 2017) its excess in plasma causes jaundice (Stillman, 1990). However, Stocker et al. (1987a) observed that BRB eliminates peroxyl radicals (ROO*) avoiding membrane lipid peroxidation, this fact, suggests that it could be used as an antioxidant molecule. Subsequent *in vivo* and *in vitro* studies showed that BRB can inactivate some ROS such as hydroperoxyl (HO2), superoxide anion ($O2^-$), hydroxyl (OH^-), (Neuzil and Stocker, 1994; Stocker et al., 1987b) and nitric oxide (NO) (Mancuso et al., 2003 and Barone et al., 2009).

Due to the bioethical limitations to perform experimentation using murine systems and even with human cells in culture (Jiménez et al., 2019), *D. melanogaster* remains as a useful alternative for the detection of substances with therapeutic potential due to the advantages it offers (Rudrapatna et al., 2012). In addition to the low maintenance cost and a short life cycle, it has been estimated that 75 % of genes related with diseases in humans have functional orthologs in the fly (Lloyd and Taylor, 2010). The purpose of this study was to evaluate the radioprotective activity of porphyrins and compare their effect with AMF® in *D. melanogaster*.

2. Materials and methods

2.1. Biological material

Two *D. melanogaster* strains were used: *mwh* + / *mwh* + and *flr*^{3}/In (3LR) TM3, *Ser* were used. Both strains have recessive genetic markers that modify wing trichomes. The *mwh* marker (multiple wing hair) is located on the left arm of chromosome 3 in position 3 − 0.3. The *flr*^{3} marker is located on the same chromosome and side, in position 3 − 38.8. The TM3 balancer is necessary since the *flr*^{3} allele is lethal in homozygous condition. The presence of the gene is confirmed with Ser (Serrate) a dominant marker gene. More detailed descriptions of the genetic markers can be found in Lindsley and Zimm (2012).

2.2. Chemical compounds

The SCC [CAS: 11006-34-1]; PP-IX, [CAS: 5865-01-5] and BRB [CAS: 635-65-4] was obtained from Sigma-Aldrich ST. Louis Missouri. AMF® (Ethyol®, 500 mg. WR-2721; Schering Plough, Levallois-Perret, France, [CAS No. 20537-88-6].

2.3. Compound concentrations

SCC concentration was selected based on previous investigations in our group, (Pimentel et al., 1999, 2000) and in this study equimolar concentrations were used based on the larvae viability: SCC 69 mM; BRB 6.9 mM (Jiménez, 2013) and PP-IX 0.69 (Vidal et al., 2014), and 32 μM of AMF® (Aydemir et al., 2009). All compounds were dissolved in a 5% sucrose solution. BRB was sonicated for 20 min with 5% sucrose solution in darkness.

2.4. Mating and larvae collection

Fourth days old *mwh* + / *mwh* + virgin females were crossed with males *flr*^{3} / *TM3; Ser*. Groups of 50 couples per bottle laid eggs for a 2 h period in 250 mL flasks with regular culture medium (agar, corn flour, sucrose, dextrose, yeast and antibiotics). Laid eggs developed in a culture room at 25 ± 1 °C and 60 % relative humidity for 3 days in order to obtain second instar larvae that were collected by difference of density with a 20 % sucrose solution.

2.5. Pre-treatment

Second instar larvae were pre-treated (PT) in flasks of ¼ L which contained a filter paper soaked with 3.5 ml of the corresponding solution: 5 % sucrose solution was used as negative control; SCC (69 mM); PPIX (0.69 mM) and BRB (6.9 mM). Duration of PT varies: 1, 3, 6 or 9 h and all the time larvae remains in culture room in total darkness. Based on results obtained by Aydemir et al. (2009) and due the pharmacokinetic of AMF®, PT with this compound only 1 and 3 h PT was tested.

2.6. Irradiation

Batch of larvae from each PT solution, was taken as non-irradiated control group and other batch for each compound was irradiated with 20 Gy of gamma rays in a Co^{60} Transelektro LGI-01 irradiator, the dose rate at the time of experiments was 699.7 Gy/h. Larvae corresponding to each pre-treatment period were placed in groups of 100 in glass vials (11 cm large and 2.5 cm diameter) containing regular medium, then they were introduce in the culture room to complete development in total darkness. At least two experiments were performed for each compound and PT duration (1, 3, 6 - or 9 h) organisms not receiving none compound and irradiated with 20 Gy served as positive control, for each one of the compounds there was a pretreatment that was irradiated PT + 20 Gy

2.7. Larvae-adult viability test

After development concluded, the number of emerged adults from each treatment was counted daily, females and males separately. The number of adults was plotted to represent the viability curves and establish the time of highest emergence of adults from different treatment.

2.8. Genotoxicity test

The SMART assay in wings of *D. melanogaster* was used. Briefly, the assay detects loss of heterozygosity of the two recessive markers that codify for the shape of the trichome in Drosophila wing cells: i.e. multiple wing hair (mwh) and flare (flr). The loss of heterozygosity in this test is the result of different genetic end points: point mutation, deletion, and mitotic recombination, as described by Graf et al. (1984). The viable individuals of each treatment were fixed in alcohol at 70 %, the wild type wings (*mwh + / + flr*^{3} genotype) were mounted on permanent slides with Faure's solutions to score the number and type of spots in a compound microscope at 400 ×. The wings were analyzed for the occurrence of single spots mwh or flr; small spots consisting of one or two cells, large single spots consisting of three or more cells, and twin spots consisting adjacent mwh and flr^3 cells. Single spots can be produced by point mutation, chromosome aberration, deletion, or mitotic recombination; twin spots originate exclusively from mitotic recombination (Graf et al., 1984).

2.9. Statistical analysis

The larva-adult viability results, were analyzed with an ANOVA test with Bonferroni adjustment at 95 % significance level in relation to the number of statistical tests performed simultaneously. The toxicity was obtained dividing the total number of viable adults between the numbers of larvae tested per 100. The genotoxicity was evaluated according to the multiple-decision procedure of Frei and Würgler (1988), which makes possible to obtain four different decisions: negative (-), weakly positive (w), positive (+) and inconclusive (i). The procedure was based in two hypotheses: (1) there is no difference in the mutation frequency between control and treatment series and (2) the pretreatment results decrease in mutation frequency. Because small single spots and total spots have a comparatively high spontaneous frequency, m is fixed at a value of 2 (testing for a doubling of the spontaneous frequency to define a negative results). For the large single spots and the twin spots, which have a lower spontaneous frequency, m = 5 is used. Both hypotheses are tested at 5% significance level. To test against the hypotheses, the conditional binomial test according to Kastenbaum and Bowman or the Chi-Square test for proportions may be applied (Frei and Würgler, 1988).

3. Results

3.1. Larva-adult viability

Fig. 1 shows the larva-adult viability percentages after PT and PT +20 Gy of gamma rays (PT +20 Gy) with the different porphyrins or AMF®. Each bar represents the percentage of viability obtained from three independent experiments. The ANOVA analyzed of larva-adult viability showed no significant differences between negative control (0) and PT with any porphyrin's exposure at different times. The comparison of the PT +20 Gy groups with the 20 Gy control group, shows that BRB provoked an increase of 16 % ($p < 0.05$) and SCC caused a reduction of 10.9 % ($p \leq 0.05$) when the larvae were exposed 3 h and 6.8 % ($p < 0.05$) with 6 h of PT.

3.2. Development time and Genotoxicity

From development time, (Fig. 2 a, b, c and d) it is observed that the highest percentage of emergence for all groups was that twelfth day. These individuals were used to evaluate the frequency of somatic mutation and recombination. Table 1 shows results obtained after PT with 1, 3, 6 or 9 h with the different porphyrins or AMF® alone, and that from combined groups (PT +20 Gy). None of the PT modify the basal frequency of any kind of spots frequencies respect to the negative control at any of the tested exposure times. Regarding combined treatments (PT + 20 Gy) compared to the 20 Gy control group, the statistical diagnosis indicated a significant decrease in small spots (+) for all compounds except for PPIX at 9 h of PT. A decrease was found for large spots, except with BRB at all exposure times tested and a weakly decreased (w) with SCC or PPIX at 9 h of PT was found.

Regarding twin spots a significant reduction (+) was obtained only for BRB at 9 h of PT. AMF® at 1 and 3 h of PT reduced the total frequency of spots (unless non statistical differences were found) but not significantly. In comparison, SCC, PPIX or BRB caused a significant reduction (+) of total spots with all PT exposure times. It highlights that the highest percentage of reduction in all exposure periods was caused with BRB (+). On the other hand, SCC and PPIX caused a weak reduction of spots with 3 and 6 h of PTs, and the exposure for 9 h with the three pigments (SCC, PPIX or BRB) caused a significant decrease (+) of the total spots.

4. Discussion

The identification of compounds with radioprotective properties, has been subject of research for several decades mainly to protect normal cells when cancer patients undergo radiation therapy (Paul et al., 2011), most of the compounds studied were derived from sulfhydryl, which are very toxic (Kouvaris et al., 2007 and Johnke et al., 2014). A plausible alternative is to identify substances from natural origin with antioxidant properties such as ascorbic acid (AA), α tocopherol and β carotene, among others. These compounds have been classified as exogenous antioxidants, which are defined as any substance whose presence in the body in concentrations lower than that of an oxidizable substrate, delays or inhibits its oxidation (Halliwell, 1999, 2006). These compounds share similar structures that includes at least one aromatic ring and one or more hydroxyl groups that can act as electron donors (Jovanovic and Simic, 1988). Ascorbic acid (AA) for example has been reported to reduce genetic damage induced by 20 Gy of gamma rays, but only when this radiation dose was administered at 32.6 and not at 836 Gy / h dose rate (González et al., 2018). Other investigations have revealed that at high concentration or in the presence of transition metals, AA can act as a pro-oxidant (Halliwell, 1999; Priéme et al., 1997). Based on the fact that they are widely distributed in nature and that they participate in fundamental processes such as photosynthesis, aerobic respiration, and metabolic activation of some chemical agents such as metallo-porphyrins, have been studied to evaluate their antioxidant and radioprotective potential. Since they are part of human metabolism, in principle they should have little or no adverse effects, among these porphyrin including chlorophylls, hemoglobin, protoporphyrin and its derivatives (Cahyana et al., 1993; Ferruzzi et al., 2001). In the present study, the protective potential of three porphyrins SCC, PPIX and BRB was compared with AMF®, (the only radioprotector approved by FDA). *Drosophila melanogaster,* an *in vivo* system that allows the relationship between various indices such as development time (DT), larva-adult viability and genotoxicity directly in the same treated individuals.

The larva-adult viability directly indicates the degree of toxicity of the tested agent. In this study it was found that neither the PT nor the PT +20 Gy modified the viability of the treated organisms compared with control groups, 0 or 20 Gy respectively, only the SCC in combination with 20 Gy, decreased the viability, while with BRB viability

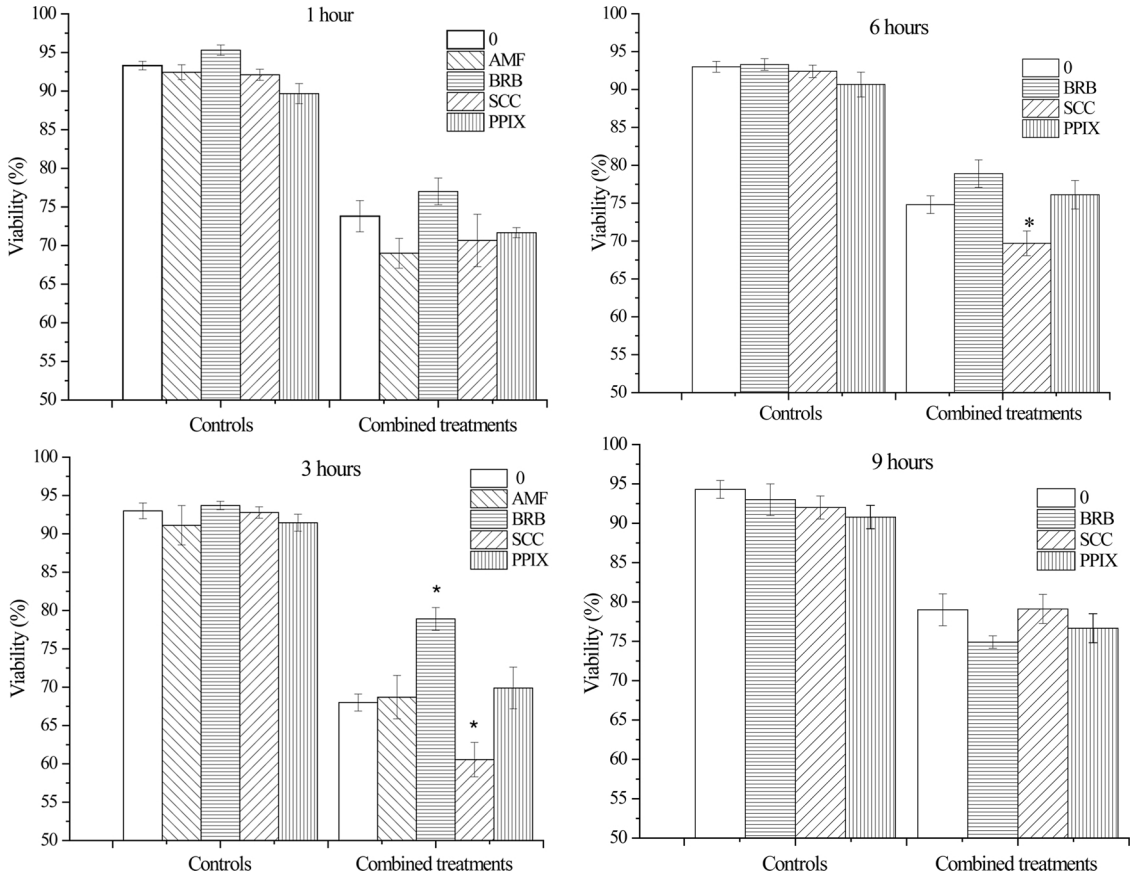

Fig. 1. Larva-adult viability of *mwh + / + flr*^{3} individuals of *D. melanogaster*, after being pre-treated with Sac (0), AMF®, BRB, SCC or PPIX (Controls) at different exposure times: 1, 3, 6 and 9 h and irradiated with 20 Gy of gamma rays (combined treatments). Each bar represents the results of three independent experiments with three replicates. Statistical analysis was performed with a Student's t: * $p \leq 0.05$.

increased 16 % with 3 h of pretreatment. Galbiati et al. (2010), demonstrated that 50, 100, and 200 mmol/L of BRB administered as a cryo-protector of human liver tissue, increased the viability and functionality of hepatocytes, and proposed BRB as an effective cryo-protectant for future transplants. An index directly related to larva-adult viability, very important when comparing different agents, is the development time (DT) of the tested individuals. This index showed that on the twelfth day (Fig. 2) was the highest percentage of adult emergence for the larvae with PT + 20 Gy, and for individuals treated only with 20 Gy. For appropriate comparisons between treatments, the flies emerged on 12 day were used to assess the potential of inhibition of genetic damage of pigments by means of the SMART assay in the *D. melanogaster* wing. None of the porphyrins or AMF® alone caused changes in mutation frequencies or recombination at any of the tested exposure times. These findings indicate that they may be even more efficient as a radio-protectors than AMF®.

Although some porphyrins such as SCC have been shown to have antimutagenic properties, particularly in reducing IR-induced genetic damage, there are others such as BRB and PPIX that, due to their similar structure with SCC, could be considered as radio-protectors. Through different experiments, it has been shown that the SCC, the most studied of the metallic center porphyrins, has the ability to form complex with mutagens that have a plane polycyclic structure (Blumer et al., 2008; Egner et al., 2003) and it can be intercalated into the DNA, preventing the mutagen from interacting with it. The copper ion of the SCC through oxide reduction reactions, is capable of donating electrons (Blumer et al., 2008) and inactivating ORSs and mutagens of oxidizing nature, thus preventing them from damaging DNA (Tumolo and Lanfer-Marquez, 2012). It has been found to be an effective antimutagen with chemo and radioprotective properties (Zimmering et al., 1990; Pimentel et al., 1999, 2000; Cruces et al., 2003; Pimentel et al., 2011), but protective properties of porphyrins without a metal center such as BRB and PPIX have been less studied.

In the present study, it was confirmed that SCC is an inhibitor of radio induced genetic damage (Cruces et al., 2009; Pimentel et al., 1999, 2013), the pre-treatment with 69 mM the percentages of reduction were similar (30.5–54.7%) to those obtained in previous reports (32 and 54 %-) but with an exposure of only 1–9 h- at 69 mM instead of 24 h as in the previous reports (Pimentel et al;, 1999; 2000). By means of the Comet assay, Gerić et al. (2019) found that pretreatment with SCC decreased the genetic damage induced by 5 Gy of gamma rays from 8.8 to 10.9%, and reduced lipoperoxidation levels from 13.1 to 16.3% in human lymphocytes.

In an earlier study by our group, it was found that 48 -h-old pretreatment of larvae for 24 h with 0.69, 6.9 or 69 mM PPIX and then treated with 0.25–2.5 mM CrO_3 -a radiomimetic chemical agent- reduced genetic damaged induced by all CrO_3 concentrations. In contrast, 6.9 and 69 mM only inhibited the damage induced by 2.5 mM CrO_3, but not the damage induced by gamma rays. These findings suggested that PPIX mainly acts by forming complexes with CrO_3 in low doses, preventing its genotoxic action, instead of capturing or inactivating reactive oxygen species, generated by this compound (Vidal et al., 2014). In the present study, 0.69 mM PPIX concentration was tested and it was found that it inhibits genetic damage induced by 20 Gy of gamma rays from 33.3 – 50.4% with 1 h –9 h, its effect was comparable to the degree of inhibition of the SCC. However, PPIX (0.69 mM) showed a higher radioprotector potential than SCC (69 mM). The importance of copper in the center of SCC as already reported Pimentel et al. (2013), it was found that the radioprotective potential of SCC depends on the percentage of copper present in the compound, samples containing 5.4 %

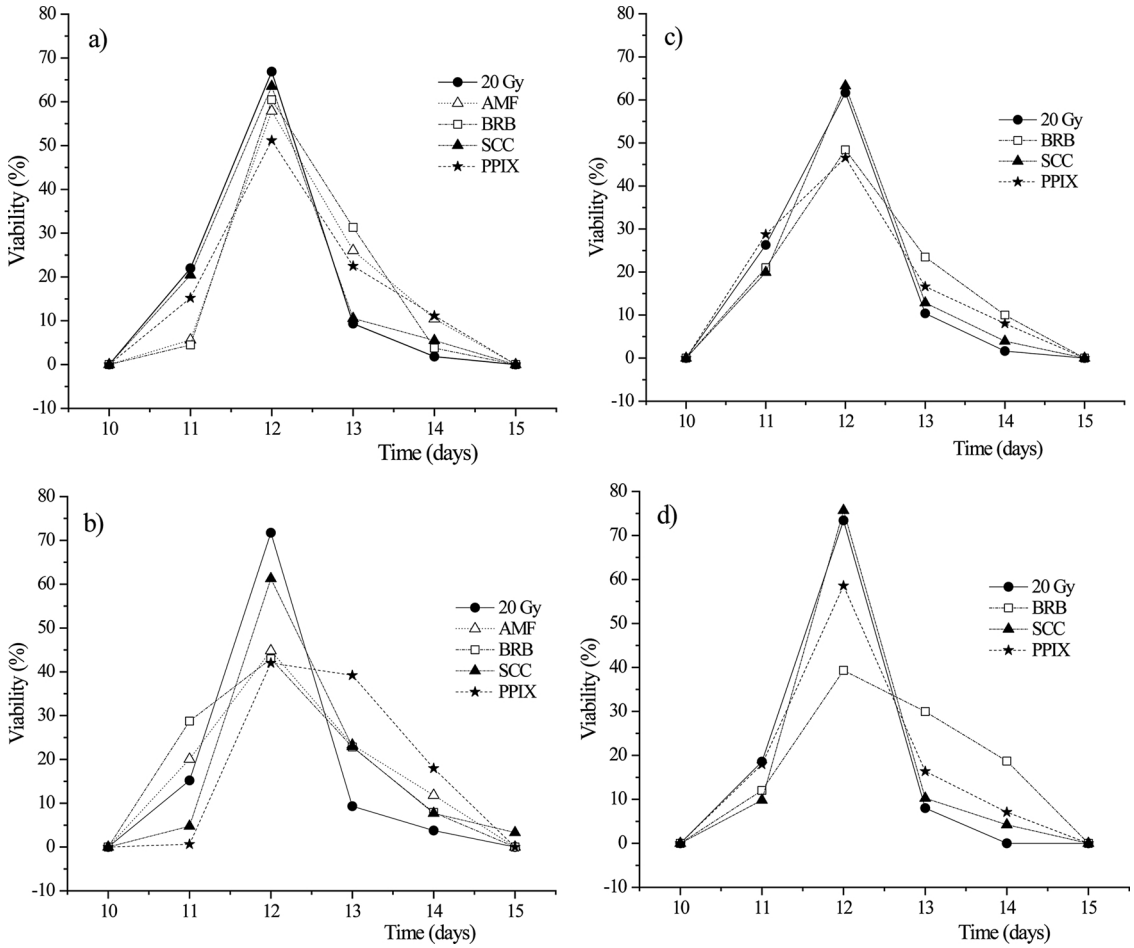

Fig. 2. Development time of *mwh + / + flr*^{3} individuals of *D. melanogaster*, after being pre-treated: a) 1 h, b) 3 h, c) 6 h and d) 9 h with AMF®, BRB, SCC or PPIX and irradiated with 20 Gy gamma rays (combined treatments).

copper caused a greater inhibition (26 %) of radiation-induced damage compared with samples containing only 3.7 %.

Its concerning BRB concentration used in the present study was determined based on a pilot experiment, where it was found that 3 h pre-treatment with 0.69; 6.9 or 69 mM BRB decreased the genetic damage induced by 20 Gy in 37.2; 59.2 and 27.6 % respectively. Therefore, it was decided to use 6.9 mM concentration of BRB for the objectives of the present study. PT for 1, 3, 6 or 9 h PT of BRB reduced the genetic damage induced by 20 Gy of gamma rays: 67.9; 71.7; 60.0 and 77.8 % respectively, in a previous experiment, it was found that 24 h of PT with 69 mM BRB decreased genetic damage caused by 20 Gy of gamma rays by more than 50 % in *D. melanogaster* and that the inhibitory effect persisted up to 72 h after the end of PT (Bailón, 2007).

Currently only amifostine® (WR-2721) has been approved for use in humans by the Food and Drug Administration (FDA) of the United States of America (Hosseinimehr, 2007). In the present investigation it was found that AMF® -caused a decrease in larva-adult viability from 1 h of pre-treatment in combination with 20 Gy of gamma rays, which was the time in which it presented its highest percentage of inhibition of genetic damage (19.1 %); with 3 h of pretreatment the percentage of viability did not change and the reduction in damage was 16.2 %, however, in both PT periods, the reduction was not statistically significant. AMF® is known to selectively protect a wide range of normal tissues, including the oral mucosa, salivary glands, lungs, bone marrow, heart, intestines, and kidneys. It offers significant protection against the nephrotoxicity, ototoxicity, and neuropathy associated with cisplatin and the hematologic toxicity associated with cyclophosphamide (Wasserman and Brizel, 2001). However, its role as an inhibitor of genetic damage was less than that of the porphyrins tested. There is evidence that AMF® reduces the mutagenic and recombinogenic effects of fotemustine -an alkylanting anticancer drug- at concentrations of 1–4 µg / ml (Aydemir et al., 2009), this action may be due to its capacity to form complex with fotemustine. Results obtained in the present study indicates that the antioxidant capacity of AMF® is reduced in Drosophila compared with the effect reported in mammals, furthermore AMF® could be a double zwitterion at physiological pH, and is poorly soluble in fat. In addition, the pH of the larva's intestine (Pimentel et al., 2013) is too low, even maybe to be compatible with the conversion to AMF® from the predecessor by the alkaline phosphatase. Evidence based on the use of special dyes shows that the food content of the midgut of *Drosophila* is strongly acidic (pH < 2.3), especially in the region of copper cells and in the stomach region (Dubreuil, 2004). A disadvantage of AMF® is its reduced radioprotection time, it must be present at the time of irradiation and its protective effect diminishes after one hour (Wasserman and Brizel, 2001). However, an important point in the present study are the averages of all the tested pretreatment times indicated similar values in larva-adult viability with was the follow relation: BRB (93.8 %) > SCC (92.3 %) > AMF® (91.8 %) > PPIX (90.6 %), the control (93.4 %) and the positive control of 20 Gy (73.9 %). Regarding radio-induced genetic damage, it was also found that porphyrins were more efficient than AMF® in reducing the frequency of mutation and recombination with the following relationship: BRB (69.3 %) > SCC (40.0 %) > PPIX (39.0 %) > AMF® (19.7 %). It is worth noting that Aydemir et al. (2009) found that AMF® reduced genetic damage induced by fotemustine in 75 % with half concentration (16 µmol / ml) used in this study (32 µmol / ml), but the drug was

Table 1
Somatic mutation and recombination frequency induced in *mwh + / + flr*^{3} individuals of *D. melanogaster* after pre-treatment in 48h-old larvae with different porphyrins or AMF® and after irradiated with 20 Gy of gamma rays.

Time of	Pre-		-SPOTS								RM	RD
Pre-	-Treat.	*n*	-Small		-Large		-Twin		Total-		(%)	(%)
-Treat	20 Gy (*)	w	#	s/w	#	s/w	#	s/w	#	s/w		
	0	80	27	0.34	6	0.07	1	0.01	34	0.42		
	AMF	80	18	0.22	13	0.16	0	0	31	0.39		
	SCC	80	22	0.27	4	0.05	2	0.025	28	0.35		
	PPIX	80	24	0.3	1	0.01	0	0	25	0.31		
	BRB	80	19	0.24	5	0.06	2	0.03	26	0.33		
1 h	20 Gy	80	65	0.81	62	0.77	3	0.04	130	1.62		
	AMF*	80	19	0.24 +	85	1.06 -	1	0.01 i	105	1.31 -	19.1	15.3
	SCC*	80	17	0.21 +	54	0.68 -	2	0.03 i	73	0.91 +	43.8	35.1
	PPIX*	80	23	0.29 +	62	0.77 -	1	0.01 i	86	1.08 w	33.3	26.6
	BRB*	80	17	0.21 +	25	0.31 +	0	0.00 i	42	0.52 +	67.9	54.3
	0	80	12	0.15	11	0.14	1	0.01	24	0.30		
	AMF	80	18	0.22	4	0.05	1	0.01	23	0.29		
	SCC	80	27	0.34+	8	0.10	3	0.04	38	0.47 i		
	PPIX	80	25	0.31	2	0.03	2	0.03	29	0.36		
	BRB	80	18	0.22	5	0.06	0	0	23	0.29		
3 h	20 Gy	80	58	0.73	80	1.00	4	0.05	142	1.77		
	AMF*	80	20	0.25 +	84	1.05 -	9	0.11 -	113	1.41 -	20.3	16.2
	SCC*	80	31	0.39 +	66	0.82 -	1	0.01 i	98	1.23 w	30.5	24.4
	PPIX*	80	18	0.22 +	73	0.91 -	2	0.03 i	93	1.16 w	34.5	27.6
	BRB*	80	18	0.22 +	20	0.25 +	2	0.03 i	40	0.50 +	71.7	57.4
	0	80	24	0.30	8	0.09	0	0	32	0.40		
	SCC	80	35	0.44	8	0.10	1	0.01	44	0.55 i		
	PPIX	80	18	0.23	7	0.09	1	0.01	26	0.32		
	BRB	80	20	0.25	5	0.06	1	0.01	26	0.32		
6 h	20 Gy	80	72	0.9	58	0.73	2	0.03	132	1.65		
	SCC*	80	32	0.40 +	56	0.70 -	3	0.04 -	91	1.14 w	30.9	24.7
	PPIX*	80	20	0.25 +	60	0.75 -	2	0.03 -	82	1.02 w	38.2	30.6
	BRB*	80	21	0.26 +	31	0.39 w	1	0.01 i	53	0.66 +	60.0	48.0
	0	80	17	0.21	2	0.02	3	0.04	22	0.27		
	SCC	80	22	0.27	5	0.06	2	0.02	29	0.36		
	PPIX	80	23	0.29	2	0.02	0	0	25	0.31		
	BRB	80	15	0.19	8	0.10	0	0	23	0.29		
9 h	20 Gy	80	46	0.57	134	1.67	7	0.09	187	2.34		
	SCC*	80	19	0.24 +	63	0.79 w	3	0.04 i	85	1.06 +	54.7	43.8
	PPIX*	80	42	0.52 -	47	0.59 w	4	0.05 i	93	1.16 +	50.4	40.3
	BRB*	80	13	0.16 +	28	0.35 +	1	0.01 +	42	0.52 +	77.8	62.2

Statistical diagnoses according to Frei and Würgler (1988): + = positive; - = negative; w = weak positive; i = unfinished, respect for control; m = multiplication factor; Probability levels: alpha = beta = 0.05. Statistical test of one tail. n: number of wings; *: combined treatments, s /w: spots per wing; RM = Reduction of mutation compared to the control of 20 Gy. RD = reduction of recombination with respect to the control according to Jiménez et al. (2019).

chronically administered to 72 h larvae until adults emerged. In the present study, exposition during only 1 − 3 h to 48 h larvae, provoked a 19.7 % reduction in the frequency of somatic mutation and recombination. Both studies confirmed the efficiency of the AMF® in Drosophila´s larvae.

Jiménez et al. (2019), determined that the percentage of recombination caused by gamma rays administered at different dose rates was around 80 %. When the relationship of the average inhibition percentage of the 4 times of PT caused by pigments was made in this study according to Jiménez et al. (2019), a relationship of the decrease in mitotic recombination was found as follows: BRB (55 %) > SCC (32 %) > PPIX (31 %) > AMF® (16 %). It is known that between 60 to 70% of the genetic damage produced by radiation is caused indirectly through the formation of FR (Shirazi et al., 2007), it can be inferred then that the action mechanism of porphyrins was probably through the inactivation of FR, which places them as potential radioprotectors for their antioxidant capacity. It has been proposed that the BRB can scavenge peroxyl radicals and hypothetically that this antioxidant mechanism was based on both the extended system of conjugate double bonds and a reactive hydrogen atom, which BRB can donate, transforming itself in a carbon centered radical (BR˙) with resonance stabilization extending over the entire molecule (Mancuso et al., 2003). Regarding SCC, its antioxidant capacity has been attributed to this capacity to stabilize various reactive species including superoxide and hydroxyl radical, by means of oxide reduction reactions donating H+ (Kumar et al., 1999). The radioprotective effect of PPIX may be because PPIX (unexcited) as BRB, inactivates FRs such as peroxyl in the dark (Williams et al., 1994).

5. Conclusion

Using the somatic mutation and recombination assay in the Drosophila melanogaster wing, it was found that pretreatment (1-9 h) with any of the porphyrins or amifostine® alone, did not affect the larva-adult viability or the basal frequency of mutation. However, they were associated with significant reductions in frequency of somatic mutation and recombination compared with the gamma rays (20 Gy) control as follows: bilirubin (69.3 %) > chlorophyllin (40.0 %) > protoporphyrin (39.0 %) > amifostine® (19.7 %). Whilst amifostine® was associated with a reduction of genetic damage after pre-treatment of 1 and 3 h, this did not attain significance. Porphyrins, in *D. melanogaster* an *in vivo* system, were more efficient in reducing genetic damage induced by 20 Gy of gamma rays compared to AMF®, which shows that PPIX, SCC and BRB may have some potential as radioprotector- agents that needs further investigation.

CRediT authorship contribution statement

E. Jiménez: Methodology, Software, Formal analysis, Investigation, Writing - original draft. **E. Pimentel:** Methodology, Software, Formal analysis, Investigation, Validation, Resources, Writing - original draft, Writing - review & editing, Visualization, Supervision, Project administration, Funding acquisition. **M.P. Cruces:** Methodology, Formal analysis, Investigation, Validation, Resources, Writing - original draft, Writing - review & editing, Visualization, Funding acquisition. **A. Amaya-Chávez:** Investigation, Writing - original draft.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

The work was in part supported by a grant No. 167461 to Cruces M.P. from the Consejo Nacional de Ciencia y Tecnología (CONACyT), Mexico, and it involved some research by Regina Jiménez Vega to obtain the Ph D. in Chemical Sciences at the Universidad Autónoma del Estado de México (UAEM) supported by CONACyT scholarship No. 334422. The authors wish to acknowledge the splendid technical assistance provided by Hugo Suarez Contreras and Ma Elena González.

References

Andreassen, C.N., Grau, C., Lindegaard, J.C., 2003. Chemical radioprotection: a critical review of amifostine as a cytoprotector in radiotherapy. Semin. Radiat. Oncol. 13, 62–72. https://doi.org/10.1053/srao.2003.50006.

Aydemir, N., Sevim, N., Celikler, S., Vatan, O., Bilaloglu, R., 2009. Antimutagenicity of amifostine against the anticancer drug fotemustine in the Drosophila somatic mutation and recombination (SMART) test. Mutat. Res.-Gen. Toxicol. Environ. 679, 1–5. https://doi.org/10.1016/j.mrgentox.2009.08.005.

Bailón, R.C., 2007. Efecto de la bilirrubina sobre el daño genético inducido por l aradiación gamma en células somáticas de Drosophila melanogaster. Tesis de Licenciatura. Toluca, Universidad Autónoma del Estado de México, Facultad de Ciencias.

Barone, E., Trombino, S., Cassano, R., Sgambato, A., De Paola, B., Stasio, D., et al., 2009. Characterization of the S-denitrosylating activity of bilirubin. J. Cell. Mol. Med. 13, 2365–2375. https://doi.org/10.1111/j.1582-4934.2008.00680.x.

Biesaga, M., Pyrzyńska, K., Trojanowicz, M., 2000. Porphyrins in analytical chemistry. Talanta 51, 209–224. https://doi.org/10.1016/S0039-9140(99)00291-X.

Blumer, A.C., Ried, K., Blanchfield, J.T., Wegner, K., 2008. The anti-mutagenic properties of bile pigments. Mutat. Res. 658, 28–41. https://doi.org/10.1016/j.mrrev.2007.05.001.

Cahyana, H., Shuto, Y., Kinoshita, Y., 1993. Antioxidative activity of porphyrin derivatives. Biosci. Biotech. Biochem. 57, 680–681. https://doi.org/10.1271/bbb.57.680.

Cruces, M.P., Pimentel, E., Zimmering, S., 2003. Evidence suggesting that chlorophyllin (CHLN) may act as an inhibitor or a promoter of genetic damage induced by chromium (VI) oxide (CrO3) in somatic cells of Drosophila. Mutat. Res.-Gen. Toxicol. Environ. 536, 139–144. https://doi.org/10.1016/S1383-5718(03)00043-3.

Cruces, M.P., Pimentel, E., Zimmering, S., 2009. Evidence that low concentrations of chlorophyllin (CHLN) increase the genetic damage induced by gamma rays in somatic cells of Drosophila. Mutat. Res. Gen. Toxicol. Environ. Mutagen. 679 (1-2), 84–86.

Dubreuil, R.R., 2004. Copper cells and stomach acid secretion in the Drosophila midgut. Int. J. Biochem. Cell. B 36, 742–752. https://doi.org/10.1016/j.biocel.2003.07.004.

Egner, P.A., Muñoz, A., Kensler, T.W., 2003. Chemoprevention with chlorophyllin in individuals exposed to dietary aflatoxin. Mut. Res.-Fund. Mol. M 523, 209–216. https://doi.org/10.1016/S0027-5107(02)00337-8.

Ferruzzi, M.G., Failla, M.L., Schwartz, S.J., 2001. Assessment of degradation and intestinal cell uptake of carotenoids and chlorophyll derivatives from spinach puree using an in vitro digestion and Caco-2 human cell model. J. Agric. Food. Chem. 49, 2082–2089. https://doi.org/10.1021/jf000775r.

Frei, H., Würgler, F.E., 1988. Statistical methods to decide whether mutagenicity test data from Drosophila assays indicate a positive, negative, or inconclusive result. Mutat. Res./Environ. Mutagen. Relat. Subj. 203, 297–308. https://doi.org/10.1016/0165-1161(88)90019-2.

Galbiati, G., Muraca, M., Mitry, R.R., Hughes, R.D., Lehec, S.C., Puppi, J., Sagias, F.G., Cruso, M., Mieli-Vergani, G.N., Dhawan, A., 2010. Bilirubin, a physiological antioxidant, can improve cryopreservation of human hepatocytes. J. Pediatr. Gastr. Nutr. 50, 691–693. https://doi.org/10.1097/MPG.0b013e3181cd26e5.

Gerić, M., Gajski, G., Mihaljević, B., Miljanić, S., Domijan, A.M., Garaj-Vrhovac, V., 2019. Radioprotective properties of food colorant sodium copper chlorophyllin on human peripheral blood cells in vitro. Mutat. Res.-Gen. Toxicol. Environ. 845, 403027. https://doi.org/10.1016/j.mrgentox.2019.02.008.

González, E., Cruces, M.P., Pimentel, E., Sánchez, P., 2018. Evidence that the radioprotector effect of ascorbic acid depends on the radiation dose rate. Environ. Toxicol. Pharmacol. 62, 210–214. https://doi.org/10.1016/j.etap.2018.07.015.

Graf, U., Wurgler, F.E., Katz, A.J., Frei, H., et al., 1984. Somatic mutation and recombination test in Drosophila melanogaster. Environ. Mut. 6, 153–188. https://doi.org/10.1002/em.2860060206.

Greer, J.P., Arber, D.A., Glader, B.E., List, A.F., Means, R.M., Rodgers, G.M., 2018. Wintrobe's Clinical Hematology. Lippincott Williams and Wilkins.

Halliwell, B., 1999. Vitamin C: poison, prophylactic or panacea? Trends Biochem. Sci. 24, 255–259. https://doi.org/10.1016/S0968-0004(99)01418-8.

Halliwell, B., 2006. Oxidative stress and neurodegeneration: where are we now? J. Neurochem. 97, 1634–1658. https://doi.org/10.1111/j.1471-4159.2006.03907.x.

Hosseinimehr, S.J., 2007. Trends in the development of radioprotective agents. Drug Discov. Today 12, 794–805. https://doi.org/10.1016/j.drudis.2007.07.017.

Jiménez, E.R., 2013. Evaluación de la modulación del estrés oxidante en cepas de Drosophila melanogaster deficientes en antioxidantes endógenos y con exposición crónica a casiopeina CII-gly y radiación gamma. Tesis de Licenciatura. Toluca, Universidad Autónoma del Estado de México, Facultad de Ciencias.

Jiménez, E., Pimentel, E., Cruces, M.P., Amaya-Chavez, A., 2019. Relationship between viability and genotoxic effect of gamma rays delivered at different dose rates in somatic cells of Drosophila melanogaster. J. Toxicol. Environ. Heal. A 82, 741–751. https://doi.org/10.1080/15287394.2019.1646681.

Johnke, R.M., Sattler, J.A., Allison, R.R., 2014. Radioprotective agents for radiation therapy: future trends. Future Oncol. 10, 2345–2357. https://doi.org/10.2217/fon.14.175.

Joshi, Y., Jadhav, T., Kadam, V., 2010. Radioprotective-A pharmacological intervention for protection against ionizing radiations. Internet J. Intern. Med. 8, 101–105. https://doi.org/10.5580/3b2.

Jovanovic, S.V., Simic, M.G., 1988. Redox properties of oxy and antioxidant radicals. Oxygen Radicals in Biology and Medicine. Springer, Boston, MA, pp. 115–122.

Kamran, M.Z., Ranjan, A., Kaur, N., Sur, S., Tandon, V., 2016. Radioprotective agents: strategies and translational advances. Med. Res. Rev. 36, 461–493. https://doi.org/10.1002/med.21386.

Kouvaris, J.R., Kouloulias, V.E., Vlahos, L.J., 2007. Amifostine: the first selective-target and broad-spectrum radioprotector. Oncologist 12, 738–747.

Kumar, S.S., Chaubey, R.C., Devasagayam, T.P.A., Priyadarsini, K.I., Chauhan, P.S., 1999. Inhibition of radiation-induced DNA damage in plasmid pBR322 by chlorophyllin and possible mechanism (s) of action. Mutat. Res.-Fund. Mol. M 425, 71–79. https://doi.org/10.1016/S0027-5107(98)00250-4.

Lindsley, D.L., Zimm, G.G., 2012. The Genome of Drosophila melanogaster. Academic Press.

Lloyd, T.E., Taylor, J.P., 2010. Flightless flies: drosophila models of neuromuscular disease. Ann. N. Y. Acad. Sci. 1184 e1. PMID: 20329357.

Mancuso, C., 2017. Bilirubin and brain: a pharmacological approach. Neuropharmacology 118, 113–123. https://doi.org/10.1016/j.neuropharm.2017.03.013.

Mancuso, C., Bonsignore, A., Di Stasio, E., Mordente, A., Motterlini, R., 2003. Bilirubin and S-nitrosothiols interaction: evidence for a possible role of bilirubin as a scavenger of nitric oxide. Biochem. Pharmacol. 66, 2355–2363. https://doi.org/10.1016/j.bcp.2003.08.022.

Mölzer, C., Huber, H., Steyrer, A., Ziesel, G., Ertl, A., Plavotic, A., Wallner, M., Bulmer, A.C., Wagner, K.H., 2012. In vitro antioxidant capacity and antigenotoxic properties of protoporphyrin and structurally related tetrapyrroles. Free Radic. Res. Commun. 46, 1369–1377. https://doi.org/10.3109/10715762.2012.715371.

Neuzil, J., Stocker, R., 1994. Free and albumin-bound bilirubin are efficient co-antioxidants for alpha-tocopherol, inhibiting plasma and low-density lipoprotein lipid peroxidation. J. Biol. Chem. 269, 16712–16719.

Odin, A.P., 1997. Vitamins as antimutagens: advantages and some possible mechanisms of antimutagenic action. Mutat. Res.-Gen. Toxicol. Eniron. 386, 39–67. https://doi.org/10.1016/S1383-5742(96)00044-0.

Paul, P., Unnikrishnan, M.K., Nagappa, A.N., 2011. Phytochemicals as radioprotective agents-a review. I.J.N.P.R. 2.

Pimentel, E., Cruces, M.P., Zimmering, S., 1999. On the persistence of the radioprotective effect of chlorophyllin (CHLN) in somatic cells of Drosophila. Mutat. Res.-Gen. Toxicol. Environ. 446, 189–192. https://doi.org/10.1016/S1383-5718(99)00182-5.

Pimentel, E., Cruces, M.P., Zimmering, S., 2000. Evidence that chorophyllin (CHLN) may behave as an inhibitor or a promoter of radiation-induced genetic damage in somatic cell of Drosophila. Mutat. Res.-Gen. Toxicol. Environ. 472, 71–74. https://doi.org/10.1016/S1383-5718(00)00125-X.

Pimentel, E., Cruces, M.P., Zimmering, S., 2011. A study of the inhibition/promotion effects of sodium-copper chlorophyllin (SCC)-mediated mutagenesis in somatic cells of Drosophila. Mutat. Res.-Gen. Tox. 722, 52–55. https://doi.org/10.1016/j.mrgentox.2011.03.001.

Pimentel, E., Vidal, L.M., Cruces, M.P., Janczur, M.K., 2013. Action of protoporphyrin-IX (PP-IX) in the lifespan of Drosophila melanogaster deficient in endogenous antioxidants, Sod and Cat. Open J. Anim. Sci. 3, 1. https://doi.org/10.4236/ojas.2013.34A2001.

Priéme, H., Loft, S., Nyyssönen, K., Salonen, J.T., Poulsen, H.E., 1997. No effect of supplementation with vitamin E, ascorbic acid, or coenzyme Q10 on oxidative DNA damage estimated by 8-oxo-7, 8-dihydro-2'-deoxyguanosine excretion in smokers. Am. J. Clin. Nutr. 65, 503–507. https://doi.org/10.1093/ajcn/65.2.503.

Rudrapatna, V.A., Cagan, R.L., Das, T.K., 2012. Drosophila cancer models. Dev. Dynam. 241, 107–118. https://doi.org/10.1002/dvdy.22771.

Sarkar, D., Sharma, A., Talukder, G., 1994. Chlorophyll and chlorophyllin as modifiers of

genotoxic effects. Mutat. Res.-Rev. Genet. 318, 239–247. https://doi.org/10.1016/0165-1110(94)90017-5.

Sarkar, D., Sharma, A., Talukder, G., 1995. Comparison of the effects of crude extract of spinach-beet leaves and equivalent amounts of chlorophyll and chlorophyllin in modifying the clastogenic activity of chromium (VI) oxide in mice in vivo. Phytother. Res. 9, 199–202. https://doi.org/10.1002/ptr.2650090309.

Sasse, A.D., de Oliveira Clark, L.G., Sasse, E.C., Clark, O.A.C., 2006. Amifostine reduces side effects and improves complete response rate during radiotherapy: results of a meta-analysis. Int. J. Radiat. Oncol. 64, 784–791. https://doi.org/10.1016/j.ijrobp.2005.06.023.

Shirazi, A., Ghobadi, G., Ghazi-Khansari, M., 2007. A radiobiological review on melatonin: a novel radioprotector. J. Radiat. Res. 48, 263–272. https://doi.org/10.1269/jrr.06070.

Stillman, A.E., 1990. Jaundice. Clinical Methods: The History, Physical, and Laboratory Examinations, 3rd edition. Butterworths.

Stocker, R., Yamamoto, Y., McDonagh, A.F., Glazer, A.N., Ames, B.N., 1987a. Bilirubin is an antioxidant of possible physiological importance. Science 235, 1043–1046. https://doi.org/10.1126/science.3029864.

Stocker, R., Glazer, A.N., Ames, B.N., 1987b. Antioxidant activity of albumin-bound bilirubin. Proc. Natl. Acad. Sci. U. S. A. 84, 5918–5922. https://doi.org/10.1073/pnas.84.16.5918.

Tomaro, M.L., del C Batlle, A.M., 2002. Bilirubin: its role in cytoprotection against oxidative stress. Int. J. Biochem. Cell B 34, 216–220. https://doi.org/10.1016/S1357-2725(01)00130-3.

Tumolo, T., Lanfer-Marquez, U.M., 2012. Copper chlorophyllin: A food colorant with bioactive properties? Food Res. Int. 46, 451–459. https://doi.org/10.1016/j.foodres.2011.10.031.

Venkatachalam, S.R., Chattopadhyay, S., 2005. Natural radioprotective agents: an overview. Curr.Org. Chem. 9, 389–404. https://doi.org/10.2174/1385272053174930.

Vidal, L.M.E., Pimentel, E.P., Cruces, M.P., Sánchez, J.C.M., 2014. Genetic damage induced by CrO3 can be reduced by low doses of Protoporphyrin-IX in somatic cells of Drosophila melanogaster. Toxicol. Rep. 1, 894–899. https://doi.org/10.1016/j.toxrep.2014.10.007.

Wasserman, T.H., Brizel, D.M., 2001. The role of amifostine as a radioprotector. Oncol.-Williston Park Then Huntington 15, 1349–1356.

Weiss, J.F., Landauer, M.R., 2003. Protection against ionizing radiation by antioxidant nutrients and phytochemicals. Toxicology 189, 1–20. https://doi.org/10.1016/S0300-483X(03)00149-5.

Williams, M., van Der Zee, J., van Steveninck, J., 1992. Toxic dark effects of protoporphyrin on the cytochrome P-450 system in rat liver microsomes. Biochem. J. 288, 155–159. https://doi.org/10.1042/bj2880155.

Williams, M., Krootjes, B.B., van Steveninck, J., van Der Zee, J., 1994. The pro-and antioxidant properties of protoporphyrin IX. Bba-Lipid. Lipid. Met. 1211, 310–316. https://doi.org/10.1016/0005-2760(94)90155-4.

Zimmering, S., Olvera, O., Hernandez, M.E., Cruces, M.P., Arceo, C., Pimentel, E., 1990. Evidence for a radioprotective effect of chlorophyllin in Drosophila. Mutat. Res. Lett. 245, 47. https://doi.org/10.1016/0165-7992(90)90024-E.