Protective effect of resveratrol against hexavalent chromium-induced genotoxic damage in Hsd:ICR male mice

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Abstract

It is well-established that exposure to hexavalent chromium [Cr(VI)] induces genotoxic damage. The aim of this study was to examine the ability of resveratrol to counteract hexavalent chromium [Cr(VI)]-induced genetic damage, as well as possible pathways that may be associated with this protection. Hsd:ICR male mice were divided into groups of 5 each and treated as follows: a) control 1, distilled water; b) control 2, ethanol 30%; c) resveratrol, 50 mg/kg by gavage; d) CrO₃, 20 mg/kg intraperitoneally; and e) resveratrol in addition to CrO₃ (resveratrol+CrO₃), with resveratrol administered 4 hr prior to CrO₃. The frequency of micronuclei (MN) and cytotoxicity were measured in peripheral blood at 0, 24, 48 and 72 hr, while 8-hydroxydeoxyguanosine (8-OHdG, 7,8-dihydro-8-oxodeoxyguanosine) adduct repair levels, endogenous antioxidant system biomarkers and apoptosis at 48 hr after treatments. Resveratrol administration increased activities of superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase (CAT). CrO₃ treatment elevated GPx and CAT activities. Resveratrol reduced the frequency of Cr(VI)-induced rise in MN and without significant effect on levels of 8-OHdG adduct when administered alone, suggesting that this polyphenol-mediated cellular repair does not involve 8-OHdG adduct formation. Concomitant administration of resveratrol and Cr(VI)-resulted in return of activities of SOD, GPx and CAT to control levels accompanied by decreased glutathione levels suggesting that the endogenous antioxidant system might play an important role in resveratrol-mediated inhibition of Cr(VI)-induced oxidant toxicity. The increase in apoptotic cell number in resveratrol+CrO₃ group as well as diminished necrosis further affirms that resveratrol effectively blocked the actions of Cr(VI).

Introduction

Hexavalent chromium [Cr(VI)] compounds are used in many industrial processes, including leather tanning, pigment manufacturing, metal plating, chromium smelting, fuel combustion, waste incineration and wood preservation (EPA 2010; Rowbotham et al. 2000). Both acute and chronic exposure to Cr(VI) compounds have been associated with the induction of carcinogenesis (Shi et al. 1999; Valko et al. 2006). The carcinogenesis of Cr(VI) may be initiated and promoted by the genotoxic damage generated during its intracellular reduction (Beyersmann and Hartwig 2008; Shi et al. 1999). The genotoxic damage induced by Cr(VI) includes formation of DNA adducts, cross-linking (DNA-protein and DNA-DNA), abasic sites, and oxidized DNA bases (DesMarais and Costa 2019; O'Brien et al. 2003). Other investigators also found that Cr(VI) plays a crucial role in induction of apoptosis, gene expression, and repair mechanisms (Mishra and Bharagava 2016; Valko et al. 2006). Although the relationship between oxidative stress and Cr(VI)-induced DNA damage remains unclear, several studies noted the enhanced generation of reactive oxygen species (ROS) and free radicals in Cr(VI)-mediated genotoxicity (Patlolla et al. 2009). Hence, substances with antioxidant properties may be considered as potential preventive and adjuvant agents in combatting oxidative stress and consequent DNA damage (de Freitas et al. 2020; García-Rodríguez et al. 2014; Sousa et al. 2021). Compounds such as polyphenols were found to exert direct effects as radical scavengers and metal chelators, as well as indirect effects through their ability to modulate the levels of transcription factors and enzymes (Gu et al. 2020; Majolo et al. 2020; Wang et al 2021).

Resveratrol (3,4',5-trihydroxy-trans-stilbene), is a polyphenol found in the stilbenes group associated with high antioxidant potential. These properties are known to be associated with its beneficial effects on health in the context of neurodegenerative and cardiovascular diseases, as well as some types of cancer, diabetes, and obesity-related disorders (Kuršvietienė et al. 2016; Nicolás-Méndez et al. 2020; Rauf et al. 2017). The antioxidant effect of resveratrol has been attributed to its ability to scavenge ROS. Studies in cell-free systems found that resveratrol (10–30 µg/ml) removed H₂O₂ (Gülçin 2012). Repossi et al. (2020) also observed that resveratrol might act through specific signaling pathways to (1) activate cytoprotective systems and repair mechanisms and (2) induce apoptosis. It is noteworthy that resveratrol was found to prevent DNA damage in bronchial epithelial cells obtained from C57BL/6J treated with house dust, a model of asthma (Zhang et al. 2020a). Quincozes-Santos et al. (2007) reported that resveratrol protected C6 glioma cell DNA damage produced by hydrogen peroxide attributed to its antioxidant properties. Burkhardt et al. (2001) noted that isolated calf thymus DNA oxidatively damaged by chromium(III) chloride was prevented by resveratrol due to an antioxidant action. Although the effect of resveratrol on metal-induced toxicity such as with arsenic trioxide, sodium arsenite, copper oxide, chromium(III) chloride and potassium dichromate have been examined in rodent heart, liver, kidneys, thymus and ovaries (Banu et al. 2016; Burkhardt et al. 2001; Chen et al. 2013; Khalid et al. 2018; Zhao et al. 2014), there are no apparent studies evaluating this polyphenol effects on Cr(VI) compound-induced genotoxicity *in vivo*. Therefore, the aim of the present study was to examine the protective effects of resveratrol against Cr(VI)-induced genotoxicity *in vivo*, and the underlying metabolic processes including 8-hydroxydeoxyguanosine (8-OHdG, 7,8-dihydro-8-oxodeoxyguanosine) adduct repair, endogenous antioxidant component system, and apoptosis, which may be involved in preventing Cr(VI)-induced DNA damage.

Materials And Methods

Chemicals and reagents

Cr(VI) (Chromium trioxide (CrO₃), purity grade 99.9%; CAS 1333-82-0), acridine orange (AO (75%); CAS 10127-02-3), ethidium bromide (EB (75%); CAS 1239-45-8), and 3,4',5-trihydroxy-trans-stilbene (Resveratrol, purity grade ≥ 98%; CAS 501-36-0) were obtained from Sigma Chemical Co. (St. Louis, MO).

Animals

Animal experiments were performed using 2- to 3-month-old Hsd:ICR male mice (weighing , 28–35 g). All mice were obtained from Harlan® at the “Facultad de Química, Universidad Nacional Autónoma de México” (UNAM), and acclimatized for two weeks prior to initiating the experiments. The animals were maintained in a temperature-controlled environment (22 °C) with a 12 hr light-dark cycle. Mice had free access to food (Purina-Mexico® small rodent chow) and water.

Study design

The evaluation criteria and work conditions were set up according to the Organization for Economic Co-operation and Development (OECD) guidelines (test number 474), Food and Drug Administration (FDA) guidelines, Environmental Protection Agency guidelines, and guidelines for the testing of chemicals specified by the Collaborative Study Group for the Micronucleus Test and the Mammalian Mutagenesis Study Group of the Environmental Society of Japan for the short-term mouse peripheral blood micronuclei (MN) test (Heddle et al. 1983; Sutou 1996).

To assess the genotoxic damage attributed to Cr(VI), a dose of 20 mg/kg CrO₃ was administered intraperitoneally. This dosage was based upon a previous study, in which this dose induced formation of MN in the peripheral blood of mice (García Rodríguez et al. 2001). Although the intraperitoneal route is an artificial exposure route, it is useful for detecting genotoxic damage in short-term protocols, such as the MN assay, when testing compounds with potential clastogenic properties. Further, this route is more sensitive and direct than inhalation or oral exposure (Hayashi et al. 1989; Sutou 1996). Thus, a short-term protocol using the intraperitoneal route of administration was selected to examine the direct genotoxic damaging effects of CrO₃.

The applied concentration of 50 mg/kg resveratrol was selected based upon the reported beneficial positive effects that similar and even higher doses (60, 100, 150, 200 or 250 mg/kg) exerted on cellular proliferation, apoptosis, invasion and metastasis, as well as tumor growth, in studies using breast, lung, pancreatic, colorectal, liver and gastric cancer cell lines (Qin et al. 2020; Wang et al. 2020; Wu et al. 2013; Xu et al. 2017; Yuan et al. 2016; Zhou et al. 2018;). In particular, Attia (2012) observed that doses of 50 or 100 mg/kg by gavage protected against genotoxic damage induced by cisplatin as evidenced by reduction in frequency of micronuclei (MN), and diminished DNA fragmentation and number of chromosomal aberrations. Based upon these findings, the effects of administration by gavage of 50, 70 and 100 mg/kg were previously evaluated. The dose of 50 mg/kg was selected as male mice administered this polyphenol concentration did not exhibit any apparent clinical signs of toxicity such as bristling hair, effects on mobility or decreased appetite.

After establishing treatment doses, the effects of resveratrol on genotoxic damage detected in CrO₃-treated mice were evaluated. These assessments were performed by MN, 8-OHdG, superoxide dismutase (SOD), glutathione peroxidase (GPx), catalase (CAT), glutathione (GSH), apoptosis, and cell viability analyses. Mice were divided into groups of 5 each and treated as follows: a) control 1, (C1); b) control 2, (C2); c) resveratrol; d) CrO₃; and e) resveratrol in addition to CrO₃ (resveratrol+CrO₃).

Two control groups were used (C1: mice treated intraperitoneally with sterile distilled water, and C2: mice treated with ethanol 30% by gavage) because the CrO₃ solution was prepared by dissolving the compound in water, whereas resveratrol was dissolved in ethanol 30%. The CrO₃ and resveratrol solutions (approximately 0.25 ml) were administered immediately after preparation. The resveratrol and CrO₃ groups were treated with a single dose of 50 mg/kg by gavage and 20 mg/kg intraperitoneally, respectively. In the group that combined resveratrol and Cr(VI) treatments (resveratrol+CrO₃), the mice

were treated with resveratrol 50 mg/kg by gavage 4 hr prior to CrO₃ intraperitoneal injection (20 mg/kg). A short-term protocol was employed based upon resveratrol bioavailability studies, which described plasma peak concentrations approximately 1 to 6 hr after treatment (Pannu and Bhatnagar 2019).

The assessment was carried out on peripheral blood obtained from the tail vein since this does not require animals to be sacrificed. For the MN evaluations, sequential peripheral blood samples of 5 µl were obtained from the same individuals, and 0-hr samples were designated also as negative control (Hayashi et al. 1994; OECD 2016). In this study, underlying metabolic processes including 8-OHdG adduct repair, endogenous antioxidant component system, and apoptosis were also evaluated because these may be involved in preventing Cr (VI)-induced DNA damage. Hence, these parameters were measured at 48 hr after treatment when the greatest genotoxic damage induced by Cr(VI) and the highest reduction MN were observed. The evaluations were carried out on the same samples of 200 µl obtained at 48 hr.

Micronuclei assay

To evaluate the frequencies of MN after treatment, peripheral blood samples (5 µl) were collected from the tail vein of each mouse every 24 hr for 4 days (0–72 hr). The samples were placed directly onto slides previously treated with acridine orange (AO) as previously described (Hayashi et al. 1990). The assessments were performed by identifying polychromatic erythrocytes (PCE), normochromatic erythrocytes (NCE), and MN in PCE using a fluorescent microscope (Nikon OPTIPHOT-2; Tokyo, Japan) with blue excitation (480 nm) and a barrier filter (515–530 nm) at 100× magnification. MN analysis was based upon 4,000 PCE per mouse, where the presence of MN was considered to indicate genotoxic damage. The relative proportion of PCE to NCE was also analyzed for 2,000 erythrocytes.

Plasma 8-hydroxydeoxyguanosine levels

The levels of the 8-OHdG adduct were measured in plasma of the same peripheral blood samples obtained at 48 hr from which the MN-PCE were evaluated. Plasma 8-OHdG levels were determined using an enzyme-linked immunosorbent assay (ELISA) using Trevigen's HT 8-oxo-dG ELISA Kit II (No. 4380-192-K; Gaithersburg, Maryland, USA), according to the manufacturer's instructions. Forty-eight hr after treatment, peripheral blood samples were collected from the tail vein of mice and plasma obtained following centrifugation. In this assay, the 8-OHdG monoclonal antibody competitively bound to 8-OHdG immobilized on pre-coated wells and in solution. Detection was performed using horseradish peroxidase-labeled conjugates and colorimetric substrates. Absorbance was measured at 450 nm. Product formation is inversely proportional to the amount of 8-OHdG present in the sample. The 8-OHdG levels were determined in duplicate (per sample) and according to the 8-OHdG standard curve. The levels of 8-OHdG are expressed as nM.

The 8-OHdG adduct was measured in blood plasma as a result of its repair by OGG1, since the sacrifice of the animals was not required. Presently, the interpretation that the excretion of the oxidized nucleosides, 8-oxodG and 8-oxoGuo in fluids such as plasma represents the rate of oxidation of guanine is accepted. This interpretation assumes that an organism is at steady state with no changes in the rate

of oxidation. In that situation, the number of oxidized guanine moieties in the nucleic acid and its precursor pool must be equal to the number removed/excreted from the cell per unit time (Poulsen et al. 2014). Thus, levels of 8-OHdG evaluated in fluids represent the balance between formation and repair rates.

Antioxidant system

The antioxidant system was evaluated by determining the activities of SOD, GPx, and CAT, as well as the GSH levels. Similarly, the assessments were carried out using the same peripheral blood samples obtained at 48 hr in which the greatest MN were measured.

Superoxide dismutase activity

Superoxide dismutase (SOD) activity was evaluated in erythrocytes using Trevigen's Superoxide Dismutase Assay Kit (Item No. 7500-100-k; Gaithersburg, Maryland, USA), following the manufacturer's instructions. Forty-eight hr after treatment, peripheral blood samples were collected from the tail vein of mice. The principle of the assay is that ions generated from conversion of xanthine to uric acid and hydrogen peroxide (H_2O_2) by xanthine oxidase converts nitro blue tetrazolium (NBT) to NBT-diformazan. NBT-diformazan absorbs light at 550 nm. SOD reduces superoxide ion (O_2^-) concentrations, thereby lowering the rate of NBT-diformazan formation. The amount of protein that inhibited NBT-diformazan to a maximum of 50% was defined as 1 U of SOD activity. The protein concentration was determined according to the instructions for the Cayman Chemicals Protein Determination Kit (Item No. 704002; Ann Arbor, MI, USA). SOD activity was measured in duplicate (per sample) and according to the SOD standard curve. The SOD activity is expressed as U/mg of protein.

Glutathione peroxidase activity

Glutathione peroxidase (GPx) activity was detected in plasma according to the instructions for Cayman Chemicals Glutathione Peroxidase Assay Kit (Item No. 703102; Ann Arbor, MI, USA) from blood samples drawn from the tail vein of mice 48 hr after treatment. In this assay, oxidized glutathione (GSSG), produced by the reduction of H_2O_2 by GPx, is recycled to its reduced state by glutathione reductase (GR) and nicotinamide adenine dinucleotide phosphate (NADPH). The oxidation of NADPH to $NADP^+$ is accompanied by a reduction in absorbance at 340 nm. Under conditions in which GPx activity is rate-limiting, the rate of decrease is directly proportional to GPx activity in the sample. One unit was defined as the amount of enzyme that oxidized 1 nmol of NADPH/min. GPx activity was determined in duplicate (per sample) and according to the GPx standard curve. The GPx activity is expressed as nmol/min/ml.

Catalase activity

Catalase (CAT) activity was evaluated in plasma according to the instructions for Cayman Chemicals Catalase Assay (Item No. 707002; Ann Arbor, MI, USA). Forty-eight hr after treatment, peripheral blood samples were collected from the tail vein of mice. This kit uses the peroxidation function of CAT to

determine enzyme activity. The method is based upon the reaction of the enzyme with methanol in the presence of an optimal concentration of H_2O_2 . The amount of formaldehyde produced was measured at a wavelength of 540 nm. One unit of CAT was defined as the amount of enzyme that induced the formation of 1 nmol of formaldehyde/min. CAT activity was measured in duplicate (per sample) and according to the CAT standard curve. The CAT activity is expressed as nmol/min/ml.

Glutathione levels

Glutathione (GSH) levels were evaluated in erythrocytes according to the instructions for Trevigen's HT Glutathione Assay Kit (Item No. 7511-100-K; Gaithersburg, Maryland, USA), which utilizes a carefully optimized enzymatic recycling method to quantify GSH. Peripheral blood samples were collected from the tail vein of mice 48 hr after treatment. During the assay, GR reduced GSSG to GSH. The sulfhydryl group of GSH reacts with 5,5'-dithiobis-2-nitrobenzoic acid to produce a yellow-colored 5-thio-2-nitrobenzoic acid (TNB) with absorbance at 405 nm, and mixed disulfide, which is reduced by GR to recycle GSH and produce additional TNB. The rate of TNB production is directly proportional to the recycling reaction, which in turn is directly proportional to the GSH concentration in the sample. The levels of GSH were determined in duplicate (per sample) according to the GSH standard curve. GSH levels are expressed as pM.

Apoptosis and cell viability

To evaluate apoptosis, necrosis, and cell viability, differential acridine orange/ethidium bromide (AO/EB) staining was performed using a technique previously adapted for peripheral blood (García-Rodríguez et al. 2013). The assays were carried out on the same peripheral blood samples obtained at 48 hr in which MN-PCE were measured. Peripheral blood samples were collected from the tail vein of mice 48 hr after treatment. Two slides were prepared per mouse, and the analysis was performed immediately. The assessments were based upon 300 cells per mouse. Necrotic and apoptotic cells were identified using a fluorescent microscope with blue excitation (480 nm) and a barrier filter (515–530 nm) at 40× magnification. Differential AO/EB staining is capable of distinguishing between viable and nonviable cells based upon membrane integrity. When the cell is viable, only the AO intercalates into DNA, making the nucleus fluoresce green; when the cell is nonviable, EB also intercalates into DNA, making the nucleus fluoresce orange, since EB overwhelms AO staining. The color of the nucleus depends on the viability of the cell, not state of the nucleus, thus nuclei in viable cells fluoresce green, whether the nucleus is healthy or apoptotic, and vice-versa. The cytotoxic effects were directly determined by assessing apoptosis, necrosis, and cell viability in peripheral blood cells.

Statistical analysis

The results of MN frequencies, the PCE/NCE ratio, viability (viable/nonviable cells), number of apoptotic and necrotic cells, levels of 8-OHdG and GSH, and activities of SOD, GPx, and CAT are expressed as mean $\pm$ standard deviation (SD). The results from the treatment groups were subjected to analysis of variance (ANOVA), followed by Tukey's post hoc test. Statistical analyses were performed using GraphPad Prism

8.0 (GraphPad Software, San Diego, CA, USA). For all analyses, the criterion for statistical significance was set at $p < 0.05$.

Results

The net induction frequency (NIF) of MN was determined using equation (1):

$$\text{NIF} = |\text{MN frequencies measured at time } x_i - \text{MN frequencies measured at time } 0| / n \quad (1)$$

where x_i is the evaluation at 24, 48, or 72 hr per group, *time 0* is the evaluation at 0 hr (before treatment) per group, and n is the number of mice per group.

The NIF was calculated to enhance the ability to determine net MN induction by eliminating baseline MN variability among treated groups at *time 0*. Fig. 1 illustrates the NIF of MN values for all treatments at 24, 48, and 72 hr after administration. Resveratrol treatment did not markedly affect frequency of MN, while the group treated with CrO_3 exhibited significantly higher MN frequency at 24, 48, and 72 hr compared with control (C1). When resveratrol was administered prior to CrO_3 treatment (resveratrol+ CrO_3 group), the MN frequency was lower compared to CrO_3 alone at all times examined (NIF 63, 50, and 47% lower, respectively).

When evaluating oxidative damage to DNA using the 8-OHdG adduct repair levels in peripheral blood plasma, resveratrol group displayed numerically higher 8-OHdG levels than control (C2), whereas CrO_3 produced lower levels than control (C1), both are not significant. However, the resveratrol+ CrO_3 group did show a significant increase in 8-OHdG levels compared to the controls (C1, C2), and CrO_3 group (Fig. 2).

The results of the enzymatic activity of SOD, GPx, and CAT are shown in Fig. 3. Resveratrol group significantly increased SOD, GPx and CAT enzymatic activities compared to control (C2). CrO_3 produced elevated GPx and CAT activities compared to control (C1), while SOD activity had a slight decrease. In the resveratrol+ CrO_3 mice, SOD, GPx and CAT activities were restored compared to CrO_3 group. In this group, SOD levels are high compared to controls (C1, C2). There were no significant differences in GSH levels in CrO_3 treated animals, but in resveratrol+ CrO_3 mice significantly lower GSH levels were noted compared to controls (C1, C2) and CrO_3 alone (Fig. 4).

Figure 5 illustrates the frequencies of healthy, apoptotic, and necrotic cells. Resveratrol did not markedly affect appearance and number of apoptotic or necrotic cells, while CrO_3 significantly induced increased number of apoptotic and necrotic cells. In the resveratrol+ CrO_3 mice, there were significantly fewer necrotic cells compared to CrO_3 and a marked change in apoptotic cells. There were no significant changes in the PCE/NCE ratio following any of the treatments compared to control groups (C1, C2), or over time 0 (Table 1). However, when measuring cell viability in nucleated peripheral blood cells, there were significantly fewer viable cells in the CrO_3 and resveratrol+ CrO_3 groups compared to control groups (C1, C2) (Fig. 6).

Table 1
PCE/NCE ratio in peripheral blood of mice treated with resveratrol and CrO₃.

Treatment	Dose (mg/kg)	Time analysis (hr)	n	PCE/NCE 1,000 cells (mean ± SD)
C1	0	0	5	48.5 ± 8.6
		24		46.7 ± 4.6
		48		48.2 ± 11.9
		72		52.0 ± 8.2
C2	60	0	5	46.7 ± 7.0
		24		47.2 ± 9.6
		48		53.0 ± 7.5
		72		43.4 ± 13.2
Resveratrol	50	0	5	54.8 ± 12.2
		24		47.5 ± 9.5
		48		48.7 ± 5.9
		72		50.5 ± 6.4
CrO ₃	20	0	5	46.5 ± 10.0
		24		45.8 ± 7.0
		48		51.1 ± 12.8
		72		39.8 ± 12.9
Resveratrol+CrO ₃	50+20	0	5	45.6 ± 7.9
		24		46.4 ± 2.8
		48		53.8 ± 12.7
		72		44.6 ± 5.8
A total of 2,000 erythrocytes were evaluated in each mouse (5 mice/group).				
C1, Control 1, vehicle only (distilled water); C2, Control 2, vehicle only (ethanol 30%);				
CrO ₃ , chromium trioxide; PCE, polychromatic erythrocytes; NCE, normochromatic erythrocytes				

No mice exposed to resveratrol or CrO₃ died. Specifically, the mice in the CrO₃ group showed clinical signs of toxicity, including bristling hair, decreased mobility and loss of appetite.

Discussion And Conclusion

The genotoxic effect of Cr(VI) was evidenced by increase in MN number which is consistent with previous findings of genotoxicity induced by Cr(VI), and particularly CrO₃ (García-Rodríguez et al. 2001; Hu et al. 2021). FDA and OECD guidelines indicate that for a substance or compound to be considered a genotoxic agent, it needs to induce more than 4 MN/1,000 PCE evaluated (Heddle et al. 1983; OECD 2016). One of the mechanisms involved in the induction of genotoxic damage by Cr(VI) is the oxidative stress generated by its reduction to trivalent chromium [Cr(III)] (Shi et al. 1999). This chemical process generates ROS, such as hydroxyl radicals ($\cdot\text{OH}$), which play an important role in oxidative damage to DNA (Jomova and Valko 2011; Hu et al. 2017). Although the mechanism underlying Cr(VI)-induced 8-OHdG formation is unclear, Xia et al. (2019) proposed that the $\cdot\text{OH}$ radical might react with 2-deoxyguanosine to form the C8-OH-adduct, which is removed as 8-OHdG, which is a well-established biomarker representing oxidative DNA damage (Gorini et al. 2021). In the present study, no significant alterations were detected in 8-OHdG levels following CrO₃-treatment. It is noteworthy that Maeng et al (2003) reported that inhalation of 18 mg/m³ sodium chromate resulted in significantly elevated 8-OHdG levels in lungs after 1 week. However, after 2 weeks exposure this dose produced no significant differences in pulmonary 8-OHdG levels with full recovery at 3 weeks, Maeng et al (2003) also demonstrated that higher inhalation sodium chromate levels did not significantly alter pulmonary 8-OHdG levels. Similarly, Thompson et al. (2012) noted that *in vitro* genotoxicity of Cr(VI) is primarily oxidative in nature at low concentrations. They observed that 8-OHdG rises to non-cytotoxic concentrations at 24 hr in cell cultures treated with different doses of sodium dichromate. It is conceivable that the intraperitoneal administration of 20 mg/kg of CrO₃ might be too high to significantly affect 8-OHdG levels in blood in agreement with the *in vivo* findings of Maeng et al 2003, and *in vitro* of Thompson et al 2012. Also, it should not be ruled out the possibility that, being a dynamic system, at the time of measurement (48 hr), 8-OHdG was already removed from blood plasma. Although an additional earlier timepoint could add value to the study, repetitive samples of 200 μl in the same organisms could affect the results. In preliminary studies with a control group (water) in which peripheral blood samples were taken every 48 hr (to allow the recovery of the organisms), it was observed that the levels of 8-OHdG did not change for 6 days. Hence the comparisons of these parameters were made against their respective control groups.

It has been proposed that Cr(VI) reduces protein expression levels for DNA mismatch repair (MMR) by inhibiting the hMLH1 and hMLH2 genes, as well as the 8-oxoguanine DNA glycosylase1 (OGG1) repair enzyme involved in base excision repair (BER) (Urbano et al. 2012; Wise and Wise 2012; Xia et al. 2019). Thus, it is possible that the observed increase of MN following CrO₃ treatment might be related to DNA repair inhibition. In the group treated with resveratrol and CrO₃, the MN frequency decreased compared to treatment with CrO₃ alone, suggesting a DNA protective effect for this polyphenol. This effect might be attributed to the antioxidant properties of resveratrol, which enables this substance to interact H₂O₂, and

superoxide ($O_2^{\cdot-}$) and $\cdot OH$ radicals (Truong et al. 2018). Previously Leonard et al. (2003) demonstrated *in vitro* that resveratrol scavenged $\cdot OH$ radicals in JB6 cells exposed to Cr(VI). Our findings showed an elevation in 8-OHdG levels in mice treated with resveratrol prior to CrO_3 . In this case two events could have occurred: (1) resveratrol reactivates repair mechanisms that had been inhibited by CrO_3 , and (2) resveratrol stimulates repair mechanisms to eliminate adduct formation induced by the oxidative damage of CrO_3 . Yan et al., (2012) observed *in vitro* that resveratrol activates the BER pathway, increasing the expression of OGG1. Further Mikuła-Pietrasik et al. (2012) noted that resveratrol enhanced the activity of the repair enzyme OGG1 in senescent human cells. In a previous study *in vivo* with (-)-epigallocatechin-3-gallate (EGCG), García-Rodríguez et al (2021) reported that 8-OHdG levels did not return to control levels when both treatments EGCG and Cr(VI) were co-administered, as found in this study. In addition, García-Rodríguez et al. (2021) detected a diminished fall in MN frequency induced by Cr(VI) similar to that observed with resveratrol. Hence, it is possible that when resveratrol was administered in combination with CrO_3 , the repair mechanisms were reactivated, contributing to the reduction in MN frequency.

Although the administration of resveratrol reduced the genotoxic damage induced by CrO_3 , by reducing the frequency of MN, polyphenol administration alone significantly increased SOD, GPx, CAT activities, as well as decreased GSH levels. Modifications on the endogenous antioxidant system might also be involved in the possible pro-oxidant effect by auto-oxidation of resveratrol. During this process, semiquinones and 4'-phenoxy, $\cdot OH$ and $\cdot O_2$ radicals are generated (Kuršvietienė et al. 2016; Sinha et al. 2016). The effects observed on the examined parameters suggested that resveratrol may help counteract the generation of these radicals, an essential activity to maintain the cellular redox balance. On the other hand, although an increase in 8-OHdG levels was observed that could be related to the possible pro-oxidant effect of resveratrol itself, this was not significant. Furthermore, the frequencies of MN were not increased in this group either. Therefore, it is suggested that resveratrol treatment is not related to oxidative DNA damage (MN induction).

In the group treated with CrO_3 , there was a fall in SOD activity accompanied by elevation in GPx and CAT activities, consistent with previous findings *in vivo* (García-Rodríguez et al. 2021). Matés (2000) proposed that SOD plays an important role as a first-line antioxidant defense enzyme by catalyzing the dismutation of the $O_2^{\cdot-}$ radical to form H_2O_2 , which is subsequently reduced to H_2O by GPx and CAT. Both *in vitro* (Wani et al. 2018) and *in vivo* studies have demonstrated the effects of Cr(VI) compounds on endogenous antioxidants such as activities of SOD, CAT, GPx, heme oxygenase-1 (HO-1), levels of GSH by oral (Wang et al. 2006) and intraperitoneal administration (García-Rodríguez et al. 2021). Although the decrease in SOD activity levels was not significant, this alterations observed with CrO_3 might be attributed to interaction of SOD with $O_2^{\cdot-}$ radicals, which are formed during the intracellular reduction of Cr(VI) to Cr(III) resulting in diminished enzymic activity. On the other hand, GSH levels were not significantly modified by CrO_3 treatment, suggesting that Cr(VI) reduction was not primarily mediated by the GSH pathway. In addition to GSH, the NAD(P)H flavoenzyme pathway in reducing Cr(VI) has been proposed, as

well as the intervention of other reducers such as ascorbate, cysteine, lipoic acid, fructose, and ribose (Valko, et al. 2006).

In the group treated with resveratrol and CrO_3 GSH levels were significantly decreased, which may be due to depletion of GSH, which is a primary antioxidant molecule that plays a fundamental role in reducing cellular oxidative stress. Further, GSH might act directly as an electron donor by eliminating $\text{O}_2^\cdot$ radicals, or through GPx catalysis by reducing H_2O_2 levels and inhibiting formation of the $\cdot\text{OH}$ radical (Franco and Cidlowski 2009). Several studies showed that polyphenols elevate GSH levels, as well as stimulate transcription of genes relevant for the synthesis of endogenous antioxidants, thus counteracting oxidative stress (Moskaug et al. 2005; Rodrigo and Gil-Becerra 2014). In the resveratrol+ CrO_3 administered group the activities of SOD, GPx and CAT were restored. This suggests that resveratrol might counteract CrO_3 -induced oxidative stress by an indirect antioxidant effect, related to regulation of the endogenous antioxidant system. In *in vitro* studies Yao et al. (2010) observed that resveratrol protected against oxidative damage induced by sodium sulfate dextran, while Chen et al. (2013) found that the polyphenol diminished damage mediated by sodium arsenite. In both cases evidence indicated that the observed protection was related to increased SOD activity. Thus, enhanced SOD activity might be related to elevation in $\text{O}_2^\cdot$ radicals generated during the intracellular reduction of Cr(VI). Banu et al. (2016) showed that 10 mg/kg resveratrol protected against potassium dichromate-induced oxidative stress in rat ovarian tissue by enhancing the activities of GPx, CAT, SOD, peroxiredoxin and thioredoxin, and lowering the concentration of H_2O_2 . Resveratrol was also found to maintain the cellular redox balance by increasing the activity of antioxidant enzymes including HO-1, CAT, GPx and SOD in rat arterial endothelial cells (Ungvari et al. 2007). Therefore, it is possible that resveratrol also removed H_2O_2 generated by SOD activity, which reduced the need for GPx and CAT activation in the group treated with resveratrol and CrO_3 . Nevertheless, one needs to consider that the endogenous antioxidant system is dynamic, and thus it is possible that our results may depend upon the evaluation time (48 hr), the dose of resveratrol used, and the experimental model.

When cell viability and apoptosis were determined, treatment with CrO_3 significantly increased apoptotic and nonviable cells. These results corroborate the cytotoxicity reported for Cr(VI) compounds (García-Rodríguez et al. 2001; 2013; Jomova and Valko 2011). The ROS generation and DNA damage induced by Cr(VI) exposure might play an essential role in cytotoxicity and the apoptotic signaling pathway (DesMarais and Costa 2019; Hu et al. 2017). The administration of resveratrol alone did not alter number of apoptotic cells. However, when polyphenol was administered prior to CrO_3 administered mice, apoptotic cell frequency increased compared to CrO_3 alone. In previous *in vitro* studies, Repossi et al (2020), observed that resveratrol induced apoptosis in cancer cells as a mechanism of elimination. In oral carcinoma squamous cells, resveratrol inhibited CBX7/Akt and activate p16 cascades (Chen et al. 2020). In breast cancer cells, Mirzapour et al (2018) showed that resveratrol elevated pro-apoptotic proteins Bcl2/Bax levels, and expression of the p53 genes and of caspases 3 and 8. Further, this polyphenol enhanced the expression of caspases 3 and 9 in gastric cancer cells (Xu et al. 2017). Hence, it is possible

that the enhanced induction of apoptosis following a combined CrO₃ resveratrol treatment may contribute to elimination of cells containing Cr(VI)-induced DNA (MN) damage. Although viable cell numbers were reduced in the group treated with resveratrol and CrO₃, this group also exhibited a significant fall in necrotic cell frequency, suggesting that polyphenol diminished cytotoxicity produced by CrO₃. Other *in vivo* studies also reported that resveratrol diminished the toxicity induced by metals such as arsenic (Yu et al. 2013; Zhang et al. 2013), copper and zinc (Zhang et al. 2020b), suggesting an involvement of oxidative stress which would contribute to maintaining the balance of the cellular redox system and reduce expression of pro-inflammatory cytokines.

Although the effect of resveratrol on heavy metal-induced toxicity was previously demonstrated (Banu et al. 2016; Burkhardt et al. 2001; Chen et al. 2013; Khalid et al. 2018; Yu et al. 2013; Zhang et al. 2013; Zhang et al. 2020b; Zhao et al. 2014), there are no apparent studies of the influence of resveratrol *in vivo* on the genotoxicity induced by metal compounds with carcinogenic potential, such as Cr (VI). Our findings demonstrated that resveratrol pretreatment decreased the MN frequency induced by CrO₃. The modulation of the endogenous antioxidant system, the elevation of the 8-OHdG adduct by activation of repair mechanisms, and the increase in apoptosis in the group treated with resveratrol prior to CrO₃, suggests that these pathways might be involved in protection of this polyphenol against genotoxic damage induced by Cr(VI), in addition to direct •OH scavenging. While resveratrol treatment modified endogenous antioxidant system constituents, this dose did not markedly alter MN frequencies, suggesting that oxidant effects are not related to MN induction or 8-OHdG adduct repair. *In vivo* studies with different doses, routes of exposure and assessment times may enable determination of specific mechanisms by which resveratrol counteracts the genotoxicity induced by Cr(VI). These studies may further clarify resveratrol potential value as a chemotherapeutic agent in prevention and treatment of diseases related to genotoxic damage, including some types of cancer.

Declarations

Author contribution Conceptualization: MCG-R. Project administration: MCG-R. Investigation: MCG-R, TN-M. Methodology: TN-M. Data analysis: TN-M, MCG-R, VMM-N, ARO-M. Writing original draft preparation: TN-M. Writing–review and editing: MCG-R, SK, TN-M. Supervision: MCG-R, SK. All authors read and approved the manuscript, and all data were generated in-house and that no paper mill was used.

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Availability of data and material The datasets/information used for this study are available upon request.

Ethics approval The Bioethics Committee of the “Facultad de Estudios Superiores-Zaragoza, UNAM” approved the experimental conditions and protocols (Code: FESZ/DEPI/363/14; FESZ/DEPI/CE/016/21).

Competing interests The authors declare no competing interests.

Code availability Not applicable.

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Figures

Figure 1

Effect of resveratrol and CrO₃ on the frequency of MN evaluated in peripheral blood of mice. Data show the MN frequency at 24, 48, and 72 hr minus the MN frequency at 0 hr (net induction frequency: NIF, see results text). The frequencies of MN in the group treated with CrO₃ increased significantly at 24, 48, and 72 hr, compared with those in the C1 group. The frequencies of MN in the resveratrol+CrO₃ group decreased significantly by 63, 50, and 47% at 24, 48, and 72 hr, respectively, compared with those in the CrO₃ group. A total of 4,000 PCE were evaluated in each mouse (5 mice/group). One-way ANOVA; $p < 0.05$: ap < vs. C1; bp < vs. C2; cp < vs. resveratrol+CrO₃. C1, Control 1, vehicle only (water); C2, Control 2, vehicle only (ethanol 30%); CrO₃, chromium trioxide; MN, micronucleus

Figure 2

Effect of resveratrol and CrO₃ on 8-hydroxydeoxyguanosine (8-OHdG, 7,8-dihydro-8-oxodeoxyguanosine) levels evaluated in peripheral blood plasma 48 hr after treatments (n = 4 mice per group). One-way ANOVA: ap < vs. C1; bp < vs. C2; cp < vs. CrO₃. In all cases p < 0.05. C1, Control 1, vehicle only (distilled water); C2, Control 2, vehicle only (ethanol 30%); CrO₃, chromium trioxide

Figure 3

(a) Superoxide dismutase (SOD), (b) Glutathione peroxidase (GPx), and (c) Catalase (CAT) activities evaluated in peripheral blood at 48 hr after treatments with resveratrol and CrO₃ (n = 5 mice per group).

One-way ANOVA: ap < vs. C1; bp < vs. C2; cp < vs. CrO3; dp < resveratrol+CrO3. In all cases p < 0.05. C1, Control 1, vehicle only (distilled water); C2, Control 2, vehicle only (ethanol 30%); CrO3, chromium trioxide

Figure 4

Average of glutathione (GSH) levels evaluated in peripheral blood erythrocytes 48 hr after treatment with resveratrol and CrO3. One-way ANOVA: ap < vs. C1; bp < vs. C2; cp < vs. CrO3 (n = 5 mice per group). In all cases p < 0.05. C1, Control 1, vehicle only (distilled water); C2, Control 2, vehicle only (ethanol 30%); CrO3, chromium trioxide

Figure 5

Effect of resveratrol and CrO₃ on frequencies of healthy, apoptotic, and necrotic cells in peripheral blood, evaluated 48 hr after treatments using differential acridine orange/ethidium bromide (AO/EB) staining. The dual fluorochrome assay is an indicator of cell metabolism and death caused by cell membrane injury. A total of 300 nucleated cells were evaluated in each mouse (5 mice/group). One-way ANOVA: ap < vs. C1; bp < vs. C2; cp < vs. resveratrol+CrO₃. In all cases $p < 0.05$. C1, Control 1, vehicle only (distilled water); C2, Control 2, vehicle only (ethanol 30%); CrO₃, chromium trioxide

Figure 6

Effect of resveratrol and CrO₃ on frequencies of viable and nonviable cells in the peripheral blood of mice evaluated 48 hr after treatments using differential acridine orange/ethidium bromide (AO/EB) staining. The dual fluorochrome assay is an indicator of cell metabolism and death caused by cell membrane injury. Nonviable cells include apoptotic and necrotic cells. A total of 300 nucleated cells were evaluated in each mouse (5 mice/group). One-way ANOVA: ap < vs. C1; bp < vs. C2. In all cases p < 0.05. C1, Control 1, vehicle only (distilled water); C2, Control 2, vehicle only (ethanol 30%); CrO₃, chromium trioxide

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