Original Research

Antioxidant and antigenotoxic role of recombinant human erythropoeitin against alkylating agents: Cisplatin and mitomycin C in cultured Vero cells

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Abstract

Cisplatin (CDDP) and mitomycin C (MMC), two alkylating agents used against various solid tumours, are a common source of acute kidney injury. Thus, strategies for minimizing CDDP and MMC toxicity are of a clinical interest. In this study, we aimed to investigate the protective role of recombinant human erythropoietin (rhEPO) against oxidative stress and genotoxicity induced by CDDP and MMC in cultured Vero cells. Three types of treatments were performed: (i) cells were treated with rhEPO 24 h before exposure to CDDP/MMC (pre-treatment), (ii) cells were treated with rhEPO and CDDP/MMC simultaneously (co-treatment), (iii) cells were treated with rhEPO 24 h after exposure to CDDP/MMC (post-treatment). Our results showed that rhEPO decreased the reactive oxygen species levels, the malondialdehyde levels and ameliorated glutathione (reduced and oxidized glutathione) modulation induced by CDDP and MMC in cultured Vero cells. Furthermore, rhEPO administration prevented alkylating agents-induced DNA damage accessed by comet test. Altogether, our results suggested a protective role of rhEPO, against CDDP- and MMC-induced oxidative stress and genotoxicity, especially in pre-treatment condition.

Keywords: cisplatin, mitomycin C, recombinant human erythropoietin (rhEPO), antioxidant property; antigenotoxic property

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Introduction

Erythropoietin (EPO) is a glycoprotein inducing the production of erythrocytes under low oxygen conditions.[1] It is synthesized predominantly by interstitial kidney and liver cells (80% and 20%, respectively) in response to tissue hypoxia.[2] EPO acts through its transmembrane receptor (EPO-R) expressed mainly on red cell precursors but also on heart and skeletal muscles, brain, kidney and vascular endothelial cells.[3–8] This glycoprotein operates primarily to stimulate erythroid cell production by supporting the survival, proliferation and differentiation of erythroid progenitor cells. Besides, another key physiological role of EPO on general tissue protection was previously confirmed.[9–14]

Recombinant human EPO (rhEPO) is a well-established drug used for treatment of anaemia which occurs with various illnesses, chiefly with the chronic kidney disease.[15] rhEPO operates via similar molecular pathways to the endogenous EPO.[16,17] Cisplatin (CDDP) and mitomycin C (MMC) are two of the most effective chemotherapeutic agents used in the management of a variety of tumours including testicular, ovarian, cervical, breast, lung, gastric and uterus.[18–23] The achievement of the therapeutic activity of CDDP and MMC is mainly limited by its nephrotoxicity.[24–28]

CDDP and MMC appear to be toxic through different mechanisms. Oxidative stress and genotoxicity were considered to be possible mechanisms implicated in CDDP and MMC induced toxicity.[29–35] Therefore, several attempts have been made to reduce CDDP- and MMC-induced nephrotoxicity by either co-administration of antioxidant and antigenotoxic compounds such as melatonin, vitamin E, selenium, curcumin and quercitin.[36–39] Thus, the need of developing an efficient nephroprotective therapy is urgently required. In this context, rhEPO is used as a promising compound in such a therapy.

In the present study, we have investigated the potential role of rhEPO to protect Vero cells against CDDP and MMC induced oxidative stress and genotoxicity.

Materials and methods

Chemicals

cis-Diamminedichloroplatinum II (CDDP) and MMC were purchased from Sigma–Aldrich, France. Experiments were performed with a commercially available preparation of rhEPO (Hemax®, Bio SIDUS S.A., Argentina). Cell culture medium (RPMI1640), fetal calf serum (FCS), phosphate-buffered saline (PBS), trypsin–ethylene diamine tetraacetic acid (EDTA), penicillin and streptomycin mixture were from GIBCO-BCL (UK), 2,4-dinitro-phenylhydrazine and guanidine were from VWR International (Fontenay-sous-Bois, France). 2′,7′-Dichlorofluorescein diacetate (DCFH-DA), 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB) and the glutathione colorimetric detection kit were from Sigma–Aldrich. All other chemicals used were of analytical grade.

Cell culture

Vero cells, from green monkey kidney[40] (Biovalori, France) were grown as monolayer culture in RPMI 1640 medium supplemented with 10% FCS, 1% L-glutamine (200 mmol/L), 1% of mixture of penicillin (100 IU/mL) and streptomycin (100 μg/mL) incubated at 37°C in an atmosphere of 5% CO_2–95 % air mixture.

CDDP was dissolved in pure dimethylsulphoxide (DMSO). To obtain the studied concentrations in the cell culture media, the drug treatment volume was negligible and represented about 0.025% of the total medium volume. MMC and rhEPO were dissolved in the cell culture medium. For this reason, we have chosen untreated cells as control.

Experimental design

In the present study, the concentrations of rhEPO and CDDP were chosen from the range of non-cytotoxic concentrations as assessed by the MTT assay (data not shown). Cells were treated with rhEPO (50 IU/mL), CDDP (60 μmol/L) and MMC (20 μmol/L) as follows:

- In pre-treatment condition:
- In co-treatment condition:
- In post-treatment condition:

Oxidative stress status

The intracellular amounts of ROS were measured by a fluorometric assay with DCFH-DA used extensively to monitor oxidation in biological systems as a well-established compound to detect and quantify intracellular products such as superoxide radical, hydroxyl radical and hydrogen peroxide.[41–43] The conversion of the non-fluorescent (DCFH-DA) to the highly fluorescent 2′,7′-dichlorofluorescein product (DCF) (λ_{max} is at 522 nm) happens in many steps. The fluorescent probe, after diffusing in the cell membrane, is hydrolysed by intracellular esterase to non-fluorescent dichlorofluorescein (DCFH), which is trapped inside the cells then oxidized to fluorescent DCF through the action of peroxides in presence of ROS.[44]

Cells (10^6 cells/well) were cultured in six-well multidishes (Polylabo, France). At 50% of confluence, Vero cells were incubated with CDDP (60 μmol/L), MMC (20 μmol/L) and rhEPO (50 IU/mL) at different treatment conditions. H_2O_2 (2 mmol/L) was used as a positive control and untreated cells were used as a negative control. They were then treated with 20 μmol/L DCFH-DA. Intracellular production of ROS was measured after 30 min incubation at 37°C by fluorometric detection of DCF oxidation on a fluorimeter (Biotek FL × 800, San Jose, CA, USA) with an excitation wavelength of 485 nm and emission wavelength of 522 nm. The DCF fluorescence intensity is proportional to the amount of intracellular ROS formation. The experiment was done in triplicate. Results are expressed as the ratio of DCF-induced treatment fluorescence/DCF-induced control fluorescence.

Evaluation of lipid peroxidation level

Lipid peroxidation was carried out by the measurement of malondialdehyde (MDA) according to the method of Ohkawa *et al*.[45] Briefly, cells (10^5 cells/mL) were cultured in 24-well multidishes (IWAKI, Asahi Glass Co, Ltd, Japan). After 24 h, cells were incubated in the presence CDDP (60 μmol/L), MMC (20 μmol/L) and rhEPO (50 IU/mL) at the different treatment conditions for 24 h followed by 1 mmol/L H_2O_2 for 2 h. Negative control cells were incubated with medium. After treatment, cells were washed with cold PBS, trypsinized, centrifuged and re-suspended in ice-cold KCl (1.15%). Samples containing 100 μL of cell lysates were combined with 0.2 mL of 8.1% sodium dodecyl sulphate, 1.5 mL of 20% acetic acid adjusted to pH 3.5 and 1.5 mL of 0.8% thiobarbituric acid. The mixture was brought to a final volume of 4 mL with distilled water and heated at 95°C for 120 min. After being cooled at room temperature, 5 mL of *n*-butanol and pyridine mixture (15:1, v/v) was added to each sample and shaken vigorously. After centrifugation at 15,000 rpm for 10 min, the supernatant fraction was isolated and the absorbance was measured at 532 nm. The experiment was done three times separately.

Glutathione assay (GSH and GSSG levels)

Reduced glutathione (GSH) and oxidized (GSSG) were measured by photometric determination. The colorimetric method for analyzing glutathione modulation is based on the GSH recycling system by DTNB and glutathione reductase. DTNB and GSH react to generate 2-nitro-5-thiobenzoic acid and GSSG. Since, 2-nitro-5-thiobenzoic acid is a yellow coloured product, GSH and GSSG concentrations can be determined by measuring absorbance. GSH can be regenerated from GSSG by GSH reductase, and react with DTNB again to produce more 2-nitro-5-thiobenzoic acid. In addition, the 5-sulphosalicylic acid was introduced for the removal of proteins from samples and for the protection of GSH oxidation.

Determination of GSH level. Cells (10^6 cells/well) were cultured in six-well multidishes (Polylabo, France) were incubated in the presence CDDP (60 μmol/L), MMC (20 μmol/L) and rhEPO (50 IU/mL) at different treatment

conditions at 37°C. Negative control cells were incubated with medium. After 24 h, cells were scrapped and collected in a lysis buffer and the salicylic acid was added for protein removal. Samples were submitted to photometric GSH determination. DTNB formation rate was monitored at 412 nm and compared with GSH standards (1–20 μmol/L). GSH levels were adjusted to total protein content determined using protein BioRad assay.[46] The experiment was done three times separately.

Determination of GSSG level. After 24 h of treatment (as described previously), cells were scrapped and collected in a lysis buffer and the salicylic acid was added for protein removal. GSSG was measured in the samples after 2-vinylpyridine addition. Samples were submitted to photometric GSSG determination. The absorbance was measured at 412 nm and compared with GSSG standards (1–20 μmol/L). GSSG levels were adjusted to total protein content determined using protein BioRad assay.[46] The experiment was done three times separately.

Single-cell gel electrophoresis (the comet assay)

DNA breaks were detected using an adaptation of the method of Singh et al.[47] Briefly, Vero cells (2×10^5 cells/well) were cultured in six-well multidishes (Polylabo, France) and treated with vehicle alone, CDDP alone (60 μmol/L), MMC alone (20 μmol/L) rhEPO alone (50 IU/mL) or rhEPO with CDDP/MMC (co-treatment, pre-treatment and post-treatment conditions). A positive control was treated with 50 μmol/L H_2O_2 for 5 min. Negative control corresponds to untreated cells. Cells were then trypsinized, centrifuged, counted and mixed with 1% (w/v) low-melting-point agarose in PBS, pH 7.4 at 37°C and immediately pipetted onto a frosted glass microscope slide pre-coated with a layer of 1% (w/v) normal melting point agarose similarly prepared in PBS. The agarose was allowed to set at 4°C for 5–10 min and the slide immersed in alkaline lysis buffer (2.5 mol/L NaCl, 0.1 mol/L EDTA, 10 mmol/L Tris, pH 10, 1% [v/v] Triton X-100 and 10% [v/v] DMSO) for 1 h at 4°C to remove cellular proteins and membranes. DNA was allowed to unwind for 40 min in the electrophoresis buffer (0.3 mol/L NaOH, 1 mmol/L EDTA, pH > 13) before horizontal electrophoresis in the same buffer for 30 min at 25 V and 300 mA. The slides were then neutralized with a Tris buffer solution (0.4 mol/L Tris-HCl, pH 7.5) for 15 min prior staining with 20 ethidium bromide (5 μg/mL). The comets were examined and scored using a fluorescence microscope. The experiment was done in triplicate. A total of 100 comets on each slide were visually scored according to the relative intensity of fluorescence in the tail and classified as belonging to one of five classes as described previously by Collins et al.[48] The total score was calculated by the following equation: (percentage of cells in class 0×0) + (percentage of cells in class 1×1) + (percentage of cells in class 2×2) + (percentage of cells in class 3×3) + (percentage of cells in class 4×4).

Statistical analysis

Each experiment was carried out at least three times separately. Data are expressed as means ± SD. Statistical comparison between different groups were done using one-way analysis of variance followed by Fischer *post hoc*, to detect the difference between various groups. Value of $P < 0.05$ was considered to be significant. (*) Indicates significant difference from the control group. (#) Indicates significant difference from the CDDP/MMC-treated cells. (a) Indicates significant difference from the co-treatment condition and (b) indicates significant difference from the post-treatment condition.

Results

Effect of rhEPO on ROS generation

To examine the changes in the redox status in response to CDDP, MMC and CDDP/MMC with rhEPO, Vero cells were exposed to the anticancer drugs and rhEPO (in the different treatment conditions) for 24 h and the production of ROS was determined by DCFH-DA assay (Figure 1). CDDP (60 μmol/L) and MMC (20 μmol/L) drug induced an important increase in ROS generation to about 4.5-fold of control in CDDP-treated cells and about 2.5-fold of control

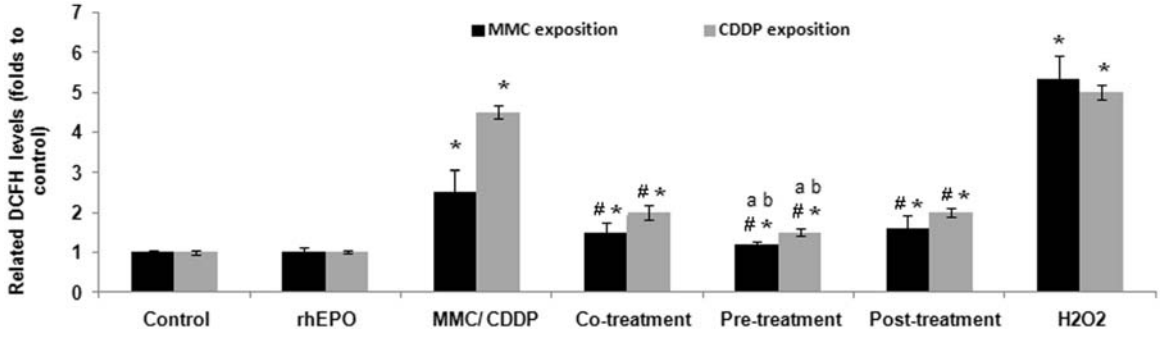

Figure 1 Levels of relative fluorescent DCF (DCF*) production in Vero cells. Different treatment conditions were performed: rhEPO (50 IU/mL) was administered simultaneously, 24 h before, and 24 h after CDDP (60 μmol/L) and MMC (20 μmol/L) exposure. H_2O_2 (2 mmol/L) was used as a positive control. Untreated cell was used as negative control. Experiment was carried out at least three times separately. Data are expressed as the mean ± SD. $^*P < 0.05$ vs. control, $^{\#}P < 0.05$ vs. CDDP/MMC, $^{a}P < 0.05$ vs. co-treatment condition, $^{b}P < 0.05$ vs. post-treatment condition

in MMC-treated cells. We deduced that CDDP caused a more pronounced oxidative damage then MMC. Conversely, cells exposed to rhEPO (50 IU/mL) in co-, pre- and post-treatment decreased the level of ROS production. ROS level did not exceed twofold of control in the presence of rhEPO with CDDP treatment. Furthermore, our results showed that pre-treatment condition promote the best protection against CDDP- and MMC-induced ROS generation in comparison with co- and post-treatment conditions. For example, in CDDP with rhEPO treatment, ROS level passed from 4.5-fold of control in the presence of CDDP to 2-, 1.5- and 2-fold of control in the presence of co-, pre- and post-treated cells, respectively.

Effect of rhEPO on lipid peroxidation induction

To evaluate lipid peroxidation status, the MDA level was measured and the results are shown in Figure 2. When compared with the control, MDA level in Vero cells treated with CDDP alone (60 μmol/L) and MMC alone (20 μmol/L) was significantly higher ($P < 0.05$). Therefore, the MDA level increased from a basal level of 6.02 ± 0.15 mmol/mg of protein in control to 18.79 ± 0.17 mmol/mg of protein in CDDP-treated cells; the increase in MDA levels was about threefold as compared with the untreated cells ($P < 0.05$). Figure 2 showed that CDDP caused a more pronounced oxidative damage than MMC. On the other hand, rhEPO administration at 50 IU/mL, whatever simultaneously, before, or after MMC, was associated with a fall in the MDA levels to reach the control value. For example, MDA level passed to 15.41 ± 0.1, 10.85 ± 0.08, and $14.88 + 0.13$ mmol/mg of protein, respectively, in co-, pre-, and post-treatment as compared with 18.79 ± 0.17 mmol/mg of protein, for CDDP-treated cells. Moreover, as shown in Figure 2, the optimum preventive effect was shown in cells treated with rhEPO 24 h before CDDP/MMC exposure.

Effect of rhEPO on glutathione (GSH + GSSG) modulation

The effect of rhEPO on CDDP and MMC induced glutathione (GSH and GSSG) modulation in cultured Vero cells is illustrated in Figure 3. Our data demonstrated that Vero cells exposed to CDDP alone and MMC alone showed a noticeable depletion of GSH level ($P < 0.05$) and a significant restoration of GSSG level ($P < 0.05$), as compared with the untreated cells. In CDDP- and MMC-treated cells GSH levels were 8.09 ± 0.3 versus 15.94 ± 1.14 and 10.11 ± 0.53 nmoles GSH/mg of protein versus 15.94 ± 1.14 nmoles GSH/mg of protein, respectively. We can deduce that CDDP caused a more pronounced oxidative damage than MMC. However, a significant increase ($P < 0.05$) in GSH level was observed in Vero cells treated with MMC/CDDP and rhEPO in different treatment conditions (co-, pre-, and post-treatment), as compared with the MMC/CDDP-exposed cells. Indeed, in CDDP-treated cells, GSH levels increased to 13.07 ± 0.53, 16.87 ± 1.01, and 12.61 ± 1.27 nmoles GSH/mg of protein, respectively, in co-, pre-, and post-treatment conditions, as compared with the CDDP treated cells (8.09 ± 0.3 nmoles GSH/mg of protein; Figure 3a). Accordingly, the GSSG levels were significantly lower in the cells treated with rhEPO in different treatment condition as compared with the MMC and CDDP treated-cells. For example, GSSG level passed from 0.45 ± 0.6 nmoles GSSG/mg of protein in the CDDP-treated cells to 0.32 ± 0.07, 0.17 ± 0.04 and 0.30 ± 0.03 nmoles GSSG/mg of protein, respectively, in co-, pre- and post-treatment conditions (Figure 3b). Further, our results demonstrated that the pre-treatment condition promoted the best protection against MMC-induced glutathione modulation and oxidative stress in Vero cells.

Effect of rhEPO on CDDP- and MMC-induced DNA damage

The potential antigenotoxic effect of rhEPO was assessed through the alkaline comet assay. Figure 4 illustrated the

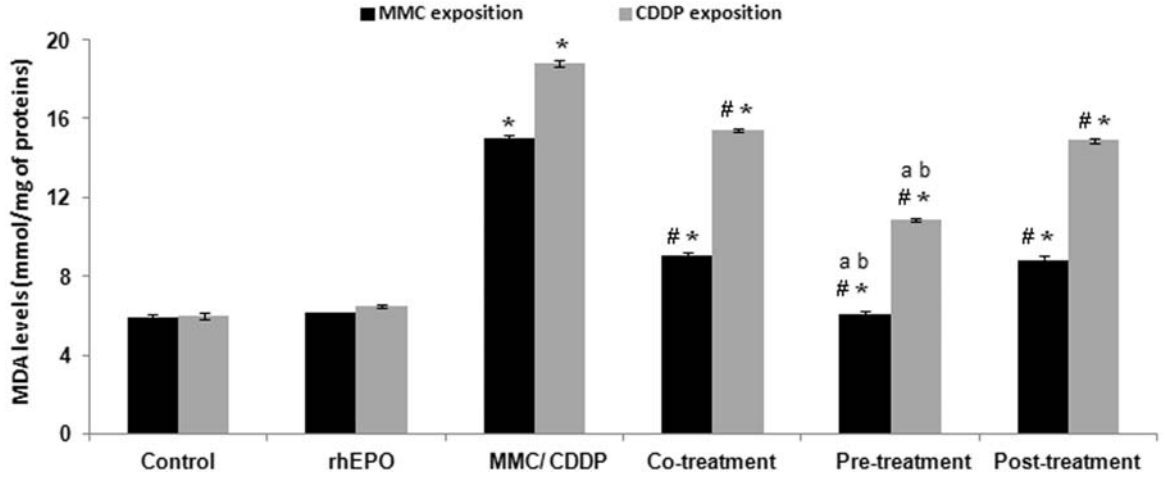

Figure 2 Lipid peroxidation as determined by MDA levels in Vero cells. Cells were treated with CDDP alone at 60 μmol/L, rhEPO alone at 50 IU/mL and CDDP (60 μmol/L) with rhEPO (50 IU/mL) at different treatment conditions (co-, pre- and post-treatment condtions). Untreated cell was used as negative control. Experiment was carried out at least three times separately. Data are expressed as the mean ± SD. $^{*}P < 0.05$ vs. control, $^{\#}P < 0.05$ vs. CDDP/MMC, $^{a}P < 0.05$ vs. co-treatment condition, $^{b}P < 0.05$ vs. post-treatment condition

five classes of comet. Result of the visual scoring of total DNA damage induced by the alkylating agents (CDDP and MMC) and prevented by rhEPO is shown in Figure 5. Our results showed that CDDP and MMC treatment at 60 and 20 μmol/L, respectively, induced a significant ($P < 0.05$) increase in DNA damage as compared with the control. Our data demonstrated that CDDP induced more marked DNA damage than MMC in Vero cells. rhEPO exposure in the different treatment conditions (co-, pre- and post-treatment) decreased significantly ($P < 0.05$) the level of DNA fragmentation as compared with the CDDP- and MMC-treated cells. The degree of DNA damage seemed to be significantly ($P < 0.05$) less noticeable when cells were pre-treated with rhEPO 24 h before CDDP and MMC intoxication as compared with co- and post-treatment. No specific DNA damage was detected in untreated cells and in cells treated with rhEPO (50 IU/mL).

Discussion

CDDP and MMC, alkylating agent, are considered as a widely used chemotherapy drugs for cancers.[18–23] Major side effects of these anticancer drugs include nephrotoxicity leading to acute kidney injury and renal failure.[24–28] Therefore, protective strategies against CDDP and MMC toxicity are necessary. The use of rhEPO has become standard for the treatment of renal and non-renal anaemia. rhEPO was reported to protect against several experimental injuries.[9–14] However, the molecular mechanism of rhEPO action against CDDP- and MMC-induced renal damage is poorly understood.

To evaluate the antioxidant property of rhEPO against alkylating anticancer drugs MMC and CDDP, we choose to check ROS production in cultured Vero cells. It has been known that ROS are essential intermediates in oxidative metabolism. Nonetheless, when oxidative stress occurs,

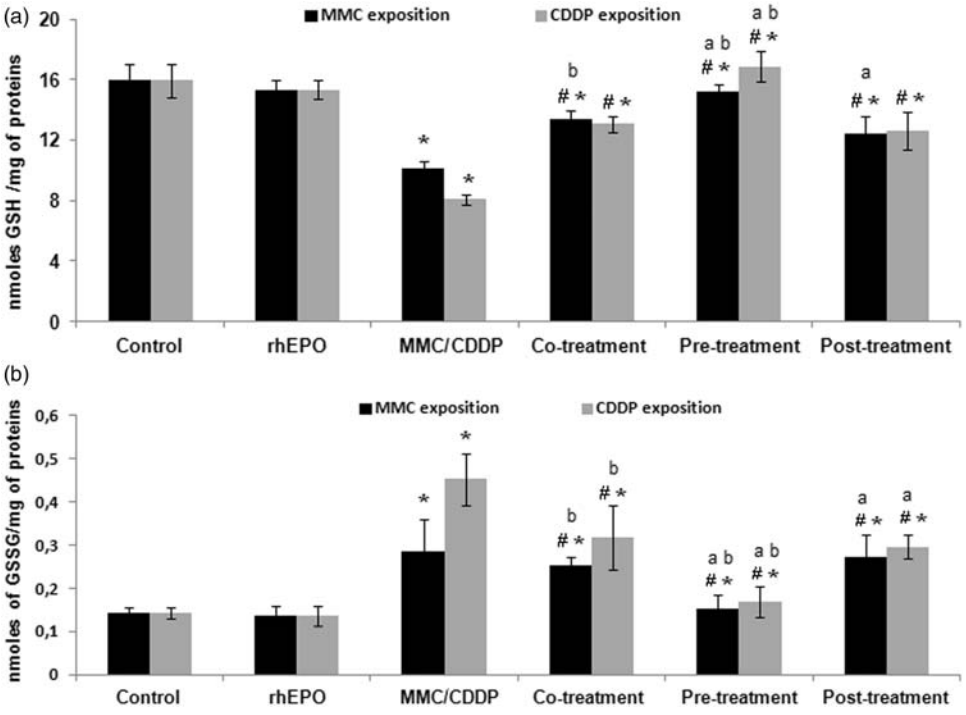

Figure 3 Effect of rhEPO administration on glutathione (a: GSH and b: GSSG) levels in treated Vero cells. Different treatment conditions were performed: rhEPO (50 IU/mL) was administered simultaneously, 24 h before, and 24 h after CDDP (60 μmol/L) and MMC (20 μmol/L) exposure. Untreated cell was used as negative control. Experiment was carried out at least three times separately. Data are expressed as the mean ± SD. *$P < 0.05$ vs. control, #$P < 0.05$ vs. CDDP/MMC, a$P < 0.05$ vs. co-treatment condition, b$P < 0.05$ vs. post-treatment condition

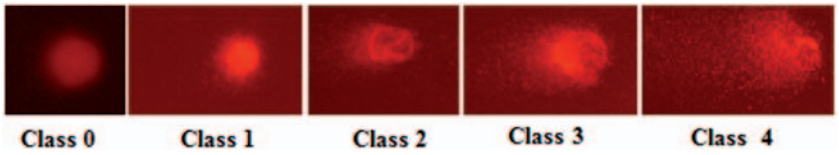

Figure 4 Examples of cells with increasing degrees of DNA damage. The class 0 is for intact DNA and class 4 is for high DNA damage. According to the intensity of fluorescence in the comet tail the DNA state is defined by five classes: 0, 1, 2, 3. (A color version of this figure is available in the online journal)

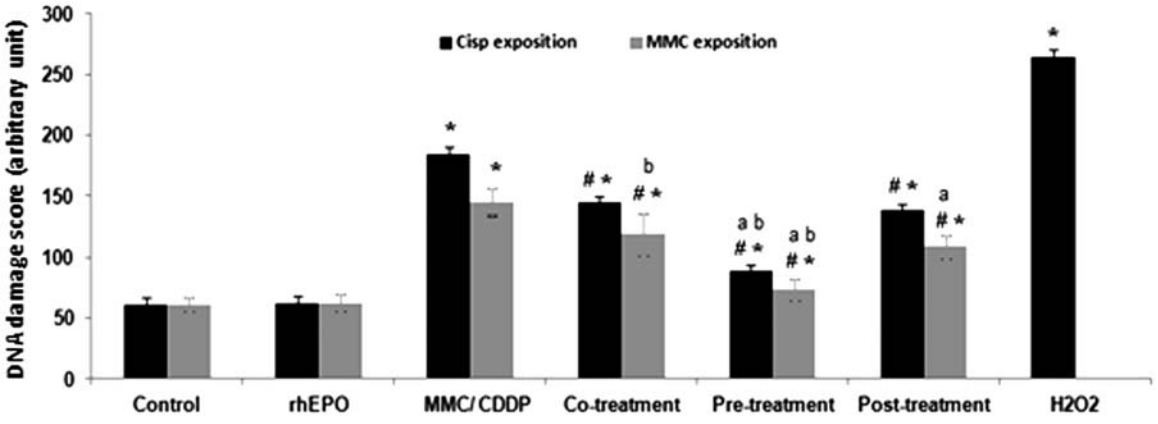

Figure 5 Total DNA damage detected by the alkaline comet assay in Vero cells. Cells were treated for 24 h with CDDP alone at 60 μmol/L, rhEPO alone at 50 IU/mL and CDDP (60 μmol/L) with rhEPO (50 IU/mL) at different treatment conditions (co-, pre- and post-treatment condtions) and assayed for DNA damage. H_2O_2 (50 μmol/L) was used as a positive control. Untreated cell was used as negative control. Experiment was carried out at least three times separately. $^{*}P < 0.05$ vs. control, $^{\#}P < 0.05$ vs. CDDP/MMC, $^{a}P < 0.05$ vs. co-treatment condition, $^{b}P < 0.05$ vs. post-treatment condition

ROS are generated in excess and consequently may damage cells by oxidizing lipids, disrupting DNA or proteins. We showed that MMC and CDDP exposure induced a noticeable increase in ROS generation as compared with control cells. Furthermore, our data demonstrated that CDDP treatment increased ROS level more than MMC treatment. rhEPO exposure simultaneously, 24 h prior and 24 h after CDDP/MMC intoxication reduced significantly the level of ROS production thus decreased the oxidative injury caused by the anticancer drugs. Statistical analysis of different treatment conditions showed that the pre-treatment with rhEPO promotes the best protection against CDDP and MMC toxicity. Our findings were in accordance with a number of few studies showing the contribution of several ROS such as O_2^-, H_2O_2 and $OH^{\cdot}$ in cytotoxicity and in cellular alterations caused by CDDP and MMC exposure *in vitro*.[29–35] Furthermore, our results were in agreement with different studies showing a protective effect of rhEPO through an antioxidant mechanisms in different cell lines.[49–52]

ROS generation caused oxidation of polyunsaturated fatty acids to produce lipid peroxylradicals and lipid hydroperoxides, a process called lipid peroxidation.[53] MDA is the end product of the major reactions, which serves as a reliable marker of oxidative stress.[54–56] So to more assess CDDP- and MMC-induced oxidative damage in Vero cells, the MDA level was examined. In the present study, while exposure to CDDP (60 μmol/L) and MMC (20 μmol/L) induced a noticeable increase in MDA formation in Vero cells, rhEPO administration either simultaneously, before or after CDDP intoxication, provided a significant reduction of this induction which dropped almost to the control level. Furthermore, our results showed that rhEPO treatment 24 h before cells intoxication with CDDP or MMC promote the best protection against the oxidative stress induced by these anticancer drugs. Our results are in accordance with those of Naziroglu *et al.*[39] who demonstrated that CDDP enhance MDA level in kidney, liver and lens of rats; and with those of Premkumar *et al.*[31] and Siddique *et al.*[57] who demonstrated that MMC exposure increased the MDA level in mice and in cultured human blood lymphocytes. Our data were also agree with Oztürk *et al.*[58] who demonstrated that EPO reduced lipid peroxidation by both decreasing NO synthesis and XO activity in rat brain injured tissue.

Oxidative stress refers a situation of a marked imbalance between the production and removal of ROS. This may be originated by an overproduction of these substances or by the depletion of antioxidant defenses.[59] The effects of rhEPO on oxidative damage caused by CDDP and MMC were also investigated through assessing glutathione (GSH and GSSG levels). Glutathione is the most abundant intracellular thiol and plays an important role in the detoxification of reactive oxygen species (ROS) and xenobiotics.[60] Our results obviously showed that CDDP and MMC decreased significantly the level of GSH and increased the GSSG level in cultured Vero cells. The amount of GSH and GSSG was clearly restored in presence of rhEPO in different treatment conditions (simultaneously, prior and after CDDP/MMC intoxication). Moreover, we clearly demonstrated that the optimum protective action of rhEPO against CDDP- and MMC-induced oxidative lesions was observed in pre-treatment condition. Our study is in agreement with those of Naziroglu *et al.*[39] who demonstrated that CDDP decrease the amount of GSH in liver and lens of rats. According to these data, we can suggest that oxidative stress is the major contributor to CDDP and MMC toxicity and rhEPO treatments can provide a good protective strategy against oxidative injury caused by these alkylating drugs.

To assess the possible antigenotoxic action of rhEPO, we monitored the comet assay which is one of the standard methods for assessing DNA damages including single- and double-strand DNA breaks.[47] Our results showed that CDDP/MMC alone caused a significant increase in DNA fragmentation as compared with the untreated cells. However, treatment of Vero cells with rhEPO simultaneously, 24 h before and 24 h after CDDP administration induced a noticeable decrease in DNA fragmentation as

compared either with CDDP- or MMC-treated cells. Furthermore, pre-treatment with rhEPO provided the best protective effect against CDDP and MMC genotoxicity as compared with co- and post-treatment. Our results are in agreement with those of Sun *et al*.[61] who showed that rhEPO (300 IU/kg intraperitoneal) administration to the rats 24 h before the insult and for two consecutive days after the insult caused a reduction in DNA fragmentation.

Our study clearly demonstrated that co-, pre- or post-treatment with rhEPO are efficient in protection against CDDP/MMC-induced oxidative stress. This protective effect was accompanied by a decrease in DNA damage caused by the anticancer drugs. It is widely known that ROS can attack biomolecules, such as DNA, lipids, and thiols, in proteins and glutathione, leading to inactivation of enzymes, genotoxic damage, cell dysfunction and death.[62,63] Many studies showed that rhEPO was directly involved in the prevention of oxidative stress with activation of antioxidant enzymes, inhibition of nitric oxide production and decrease of lipid peroxidation.[49–52] For this, the antioxidant action of rhEPO can inhibit the genotoxic effect of CDDP/MMC. It is of note that, pre-treatment with rhEPO 24 h before intoxication procured the most efficient protective effect.

The present study could contribute to the understanding of the mechanism by which rhEPO inhibits alkylating anticancer drugs toxicity in Vero cells and point towards rhEPO clinical implications in cancer therapies.

Author contributions: Dr KRT designed the study, conducted the study and wrote the manuscript. Dr IAB supervised the study and conducted statistical analysis. Mr NS conducted statistical analysis and Dr SA revised the manuscript. All authors have read and approved the final manuscript.

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