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Research Article

Application of Comet Assay to Assess the Protective Effect of Amifostine on Doxorubicin-Induced DNA Damage in Human Hepatoma Cells

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Abstract

Doxorubicin is an anthracycline anticancer drug effective against many human malignancies. Several mechanisms have been proposed for the antitumor effects of doxorubicin, such as DNA synthesis inhibition, DNA binding and alkylation, DNA crosslinking, inhibition of topoisomerase II, free radical generation and lipid peroxidation. Amifostine, is a cytoprotective adjuvant used in cancer chemotherapy, involving DNA-binding chemotherapeutic agents. The aim of this study was to explore whether amifostine protects against doxorubicin-induced genotoxicity in HepG2 cell line. For this purpose, we measured the DNA damage level with comet assay in HepG2 cells treated with doxorubicin and amifostine in different experimental conditions. We also measured the intracellular ROS generation and GSH levels in cells treated with doxorubicin and amifostine in pre-treatment condition. Our results showed that doxorubicin induced a noticeable genotoxic effect in HepG2 cells. Amifostine reduced the effects of doxorubicin significantly ($p < 0.0001$) by reduction of the level of DNA damage via blocking ROS generation, and enhancement intracellular glutathione levels.

Keywords: *Doxorubicin; Amifostine; Comet Assay; ROS; Genotoxicity*

Introduction

In cancer treatment, doxorubicin (DOX) is a commonly used drug against several human malignancies such as leukemia, lymphoma and other solid tumors [1,2]. A major adverse side effect associated with DOX usage in the clinic is the cardiomyopathy and heart failure [3]. Several mechanisms have been proposed for the antitumor effects of DOX, such as DNA synthesis inhibition, DNA binding and alkylation, interference with DNA strand separation, inhibition of topoisomerase II, free radical generation and lipid peroxidation [4,5]. DOX is genotoxic in the heart and the DNA damage may be induced primarily via the production of reactive oxygen species [6]. As an anthracycline, DOX is known to intercalate into DNA *in vitro,* and several crystal structures of complexes of DNA with DOX exist [7,8]. In several *in vitro* studies, DOX was shown to cause DNA breaks and to interfere with DNA synthesis [9]. Another investigation has shown that the DNA-DOX interaction is related to the poisoning of topoisomerase II (TOP2A), but not topoisomerase I [10]. Translocation of DOX into the nucleus is thought to occur via binding to proteasomes. Subsequent TOP2A poison-mediated cytotoxicity is considered to involve the mismatch repair genes MSH2 and MLH1 because the loss of DNA mismatch repair function results in resistance to doxorubicin [11]. Topoisomerase II-mediated DNA damage is followed by cell death [12]. TP53, a gene that has a main role in the DNA-damage response and apoptosis, has been involved in DOX-apoptosis pathway [13,14].

Several studies have shown an up regulation of TP53 occurs with anthracycline treatment, and ERCC2 and TP53 have been shown to functionally interact in a p53-mediated apoptotic pathway with DOX treatment in lymphoblastoid cell lines [14-16]. DOX can undergo a one-electron reduction by several oxidoreductases to form a DOX-semi Quinone radical [17]. These enzymes include mitochondrial NADH

dehydrogenases present in the sarcoplasmic reticulum and mitochondria [18]. Therefore, Doxorubicin has shown a range of genotoxic effects in normal cells including mutation induction and inhibition of DNA synthesis. The genotoxic effects of DOX have been proven in chromosome aberration tests, micronucleus assays and comet assay [19-21]. In this context, strategies to protect against DOX-induced genotoxicity are of clinical interest and cyto-protective agents are essential to provide this protection.

Amifostine (AMF, WR-2721), is a cytoprotective adjuvant used in cancer chemotherapy and radiotherapy involving DNA-binding chemotherapeutic agents [22]. Amifostine is an organic thiophosphate prodrug which is hydrolyzed *in vivo* by alkaline phosphatase to the active cytoprotective thiols metabolite, WR-1065 [23,24]. The selective protection of non-malignant tissues is believed to be due to higher alkaline phosphatase activity, higher pH and vascular permeation of normal tissues [25]. Amifostine is an inactive prodrug that cannot protect cells until dephosphorylated to the active metabolite, WR-1065, by alkaline phosphatase in the plasma [26]. According to the several reports, inside the cell, amifostine's protective effects appear to be mediated by scavenging free radicals, hydrogen donation, induction of cellular hypoxia, the release of endogenous nonprotein sulfhydryl's (mainly glutathione) from their bond with cell proteins, the formation of mixed disulphides to protect normal cells [27].

The WR-1065 has shown remarkable radio and chemo protective effects *in vitro* and *in vivo*. It is currently approved for clinical use as a protective agent against renal toxicity induced by cisplatin in patients being treated for ovarian cancer and against xerostomia induced by ionizing radiation in patients with head and neck cancer [28-31]. Preclinical studies have shown that administration of WR-2721 before irradiation protected against radiation clastogenesis, mutagenesis and carcinogenesis [32,33]. Amifostine is able to inactivate electrophilic substances and scavenge free radicals [34]. Besides results obtained from several studies has been showed that amifostine protects against cardiotoxicity, nephrotoxicity and genotoxicity result from chemotherapy agents [29,35-37].

Single cell gel electrophoresis (comet assay) is widely used in genotoxicity testing and is also becoming an important and sensitive tool for evaluating genotoxic potential of compounds such as mutagens and/or antimutagenic agents and carcinogens *in vivo* and *in vitro*. In the comet assay, induced DNA damage is evaluated after single cell gel electrophoresis by measuring the tail moment as the product of percent tail DNA multiplied by the tail length of the comet and the percent head DNA. After alkaline lysis, damaged DNA originating from DNA strand breaks and alkali-labile sites thereby pass out of the nuclei moving towards the anode along the electrical field and form comet-like structures [38].

The aim of present study was to investigate the protective effect of amifostine against DOX induced genotoxicity. For this purpose, we measure the DNA damage level with comet assay in HepG2 cells treated with DOX and amifostine in different experimental conditions. We also measured intracellular ROS generation and GSH levels in cells treated with DOX and amifostine in pre-treatment condition.

Material and Method

Chemicals

Doxorubicin was purchased from sigma-Aldrich, France. Amifostine, EDTA, H_2O_2, NaCl, NaOH, Na_2CO_3, NaH_2PO_4, Tris, and Triton X-100 were acquired from Merck Co. (Germany). Low melting point agarose (LMA), Na_2HPO_4, KCl and ethidium bromide were from Sigma Co. (USA). Normal melting point agarose (NMA) was supplied by Cinnagen Co (Germany). The RPMI 1640 medium, fetal bovine serum (FBS) and the antibiotic were purchased from biosera (France). DCFH-DA probe and mBCl were from sigma Aldrich (USA) And, HepG2 cells came from Pasture Institute (Iran). All other chemicals used were of analytical grade.

Cell culture

Human hepatoma (HepG2) cells were obtained from Pasture Institute of Iran were grown as monolayer culture in RPMI 1640 medium supplemented with 10% FBS, 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. Amifostine was dissolved in the cell culture medium. We have chosen untreated cells as a control. Cells were seeded in 24-well culture plates at 25×10^4 cells/well, after overnight growth, cells treated with studied concentrations of amifostine (1,5 and 10 mg/ml) 2 h prior and Simultaneously to DOX treatment (1 µM) for 1h at 37°C [38].

Single-cell gel electrophoresis (SCGE, the comet assay)

The comet assay procedure has been described in our previous studies Briefly, incubated cell suspensions (1×10^6 cells/ml) were mixed with 1% LMP agarose at 37 ºC, were placed on the precoated slides (1% NMP agarose), and covered by cover glasses for 5 min at 2 - 8°C. The slides were incubated with lysis solution (pH = 10.0) for 40 minutes and rinsed with distilled water to remove the excess lysis solution. In the next step, slides were incubated with electrophoresis buffer (pH > 13.0) for 40 minutes. Electrophoresis was conducted for 40 min at 25 V with an electricity current adjusted to 300 mA. After this stage, the slides were rinsed with distilled water to remove excess alkaline buffer and were placed in the neutralization solution (pH = 7.5) for 10 minutes. The slides were covered by sufficient dye solution (20 µg/ml ethidium bromide) for 5 min and washed with distilled water. Finally, comets were visualized under × 400 magnification using fluorescence microscope with an excitation filter of 510 - 560 nm and the barrier filter of 590 nm All stages of comet assay were performed in dark conditions and all solutions were prepared freshly and used cool.

Measurement of Oxidative Stress

Approximately 4×10^4 cells per well were cultured for 24h in 96-well plates (black-wall/clear-bottom). Thereafter, the medium was aspirated, and the cells were washed twice with HBSS. The cells were then treated with studied concentrations of amifostine (1, 5 and 10 mg/ml) 24h prior DOX treatment (1 µM) for 1h at 37 ◦ C. After the treatment, cells were washed twice with HBSS and incubated in 2 ml of fresh culture medium without FBS. 2-, 7- Dichlorodihydrofluorescein diacetate was added at a final concentration of 10 µM and incubated for 20 min. The cells were then washed twice with PBS and maintained in 1 ml of culture medium. Assess ROS by immediately analyzing cells by fluorescence plate reader using the 488 nm for excitation and detected at 535 nm. we have chosen untreated cells as a negative control and cells treated with 0.1 mM H_2O_2 as a positive control [38].

Measurement of intracellular GSH levels

HepG2 cells were plated in a 96-well plate at 50,000 cells/well. After overnight growth, they were treated with test vehicles and then incubated with monochlorobimane (mBCI, 40 µM) in a staining solution (5mMglucose, 1 mM CaCl2, 0.5 mMMgSO_4, 5 mg/ml BSA) for 30 min at 37°C in the dark. Although mBCI is a non-fluorescent probe, it forms a stable fluorescent adduct with GSH in a reaction catalyzed by the GSH S-transferases. The mean fluorescent intensity of the fluorescent GSH-bimane adduct was measured using a Spectra fluorescent plate reader at λex = 380 nm and λem = 460 nm to detect GSH. The assay was performed for amifostine for studied concentration (1,5 and 10 mg/ml) and DOX (1 µM) in pretreatment condition [38].

Statistical analysis

Tail moment (percentage of DNA in the tail × tail length), tail length (the length of the comet tail), and percent of DNA in the tail (percentage of colored spots in tail) are the most frequently used factors in the evaluation of DNA damages in the comet assay method. We used these factors for statistical analysis in this investigation. One-way analysis of variance (ANOVA) followed by Tukey's multiple comparison post hoc tests was used to compare the results of all assays. Value of $p < 0.05$ was considered to be significant.

Results

Study the effect of amifostine on DOX-induced DNA damage

The anti-genotoxic effect of amifostine was investigated through the alkaline comet assay. Results of the visual scoring and percentage of total DNA damage induced by DOX and prevented by amifostine were shown in table 1. We observed that DOX treatment at 1 µM induced a significant ($p < 0.001$) increase in DNA damage as compared to the control group. Amifostine in the different treatment conditions decreased significantly ($p < 0.0001$) the level of DNA fragmentation as compared to the DOX group.

	Treatment	Tail length (Pixels) (Mean ± SEM)	%DNA in Tail (Mean ± SEM)	Tail moment (Mean ± SEM)
Pre-treatment	Control (DOX 10 µM)	117.4 ± 3.8	58.86 ± 1.2	51.9 ± 3.23
	Amifostine (1 mg/ml)	70.43 ± 1.6	45.6 ± 1.2	44.6 ± 1.3
	Amifostine (5 mg/ml)	20.58 ± 1.06*	7.3 ± 0.2*	3.4 ± 1.5*
	Amifostine (10 mg/ml)	12.24 ± 1.35*#	3.2 ± 0.5*#	0.6 ± 0.083*#
Co-treatment	Control (DOX 1µM)	117.4 ± 3.8	58.86 ± 1.2	51.9 ± 3.23
	Amifostine (1 mg/ml)	85.2 ± 1.5	59.68 ± 1.7	54.43 ± 1.2
	Amifostine (5 mg/ml)	9.8 ± 1.6*	14 ± 0.31*	9.8 ± 1.6*
	Amifostine (5 mg/ml)	19.14 ± 1*	6.1 ± 0.4*	1.6 ± 0.05*

Table 1: *The Geno protective effect of Amifostine compared with control groups on tail length (pixels), percentage of DNA in tail, and tail moment (pixels) that are represented as mean* ± SEM. * and # mean value was significantly different from control and co-treatment group ($p < 0.0001$) (one-way ANOVA followed by turkeys post hoc test).

Study the effect of amifostine on ROS generation in DOX-treated cells

To investigate the role of oxidative stress in DOX-induced genotoxicity, we used DCFH-DA, a cell-permeable fluorescent dye, to examine the ROS generation in HepG2 cells in response to DOX stimulation. Incubation with DOX for 1h showed a considerable increase in oxidant-induced 2-, 7-dichlorofluorescein fluorescence in HepG2 cells (Figure 1). H_2O_2-mediated DCF fluorescence occurred after 1h incubation with DOX (1 µM) in HepG2 cells. This suggests that DOX, induce intracellular oxidative stress, involved in its genotoxicity. After that cells were treated with amifostine in pre-treatment condition and subsequently examined. Amifostine was significantly ($p < 0.0001$) reduced ROS generation as compared to the DOX group. Untreated cells served as control

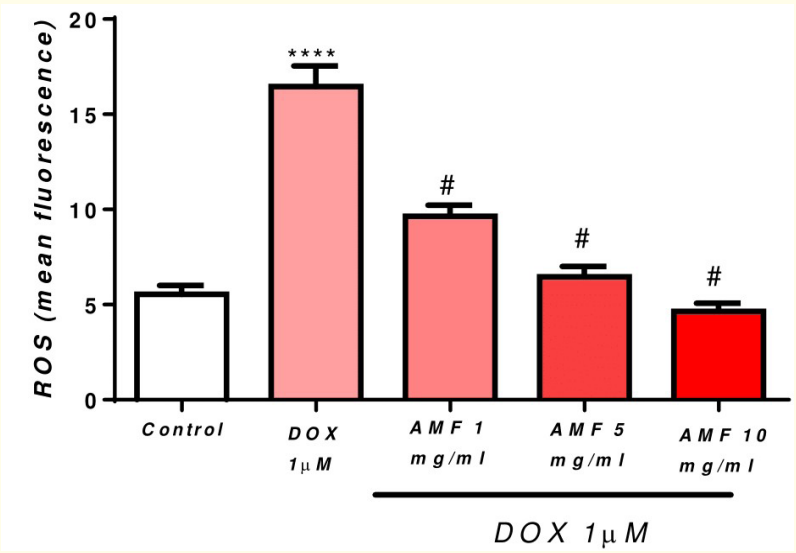

Figure 1: *Study the effect of amifostine on DOX-induced ROS generation. (****) show significantly increased results (respectively $p < 0.0001$) as compared to the control group. The sign (#) show significantly ($p < 0.0001$) decreased compared to the DOX group.*

Study the effect of DOX on intracellular levels of GSH

We first examined the effect of DOX on the intracellular levels of GSH using mBCI which readily enters cells to form a fluorescent GSH-bimane adduct that can be measured fluorometrically. As shown in figure 2, within 1h after DOX (1 μM) treatment, the intracellular levels of GSH were reduced ($p < 0.0001$). This finding was subsequently confirmed by an enzymatic assay using glutathione reductase and 2-vinylpyridine. Next, we measured the intracellular levels of GSH in cells after treatment with amifostine and DOX in pre- treatment condition. As shown in figure 2 amifostine were significantly ($p < 0.0001$) increased GSH levels as compared to the DOX group.

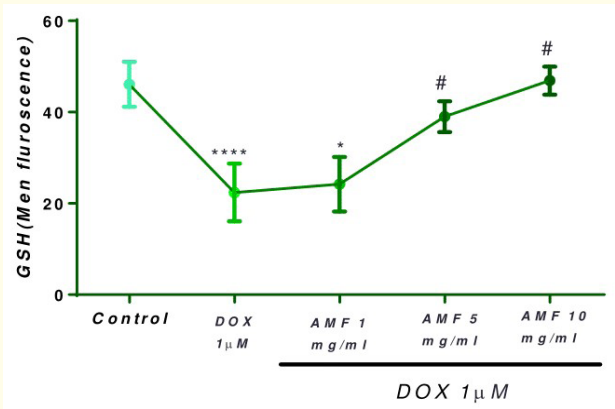

Figure 2: *The effect of amifostine on the levels of intracellular GSH were determined .ANOVA analysis revealed that amifostine, significantly inhibited the effects of DOX on the levels of GSH. Sign (****) and (*) show significantly decreased results (respectively $p < 0.0001$ and $p < 0.05$) as compared to the control group. Sign # show significantly ($p < 0.0001$) increased as compared to the DOX group.*

Discussion

In cancer treatment, DOX is a commonly used drug against several human malignancies such as leukemia, lymphoma and other solid tumors [2,39]. A major adverse side effect associated with DOX usage in the clinic is the cardiomyopathy and heart failure [40]. Several reports suggest that DOX-induced apoptosis plays an important role in its cardiotoxicity that is linked to the formation of reactive oxygen species (ROS) derived from redox activation of DOX [6]. Recent studies have focused on DOX-induced apoptotic signaling mechanisms. Several mechanisms have been proposed for the genotoxic effects of DOX, such as DNA synthesis inhibition, DNA binding and alkylation, interfering with DNA strand separation, inhibition of topoisomerase II, free radical generation and lipid peroxidation [4,17]. Previous studies have been shown that DOX induced apoptosis in normal cell types and tumor cells via different mechanisms. In endothelial cells and cardiomyocytes, DOX induced apoptosis by H_2O_2-mediated mechanism and is independent of the p53 activation. In contrast, p53 tumor suppressor, and not H_2O_2, plays a critical role in inducing apoptosis by DOX in tumor cells [14]. Therefore, our study had three general aims. Firstly, we tried to assess the ability of Doxorubicin to damage DNA in human hepatoma cells. Secondly, we explored the protective effect of amifostine against DNA-damaging effects evoked by DOX. Thirdly, we attempted to evaluate the protective potential of amifostine against generation of ROS and depletion of intracellular glutathione levels as the probable genotoxic mechanism. Our experimental data indicate that DOX can generate damage to DNA in HepG2 cells ($p < 0.0001$). It is likely, that the damage is caused by oxygen radicals generated by DOX; DNA methylation by the drug can also contribute to the damage.

Amifostine, is the most effective radioprotector known and the only one accepted for clinical use in cancer radiotherapy [41]. This antigenotoxic effect was explained by assuming a high affinity of amifostine for DNA, thereby stabilizing the DNA molecule and facilitating the activity of DNA repair enzymes [42]. Previous studies using mammal cells have shown that amifostine enhances DNA repair and thus improves cell survival. Amifostine phosphorylated aminothiol, also is an antioxidant clinically prescribed to prevent the neutropenia-associated events in patients receiving alkylating agents [43]. In experimental animals, Yuhas and Storer showed that treatment with AMF effectively protects normal tissue from the toxicity of therapeutic radiation, without protecting tumor [44]. Nagy., *et al.* subsequently showed that AMF showed the protective effect against the mutagenicity of cisplatin, evaluated by the mutation rate of HPRT in V79 Chinese hamster cells [45]. Other reports documented that amifostine protects normal tissue against radiation-induced damage by increasing intracellular SOD2 activity. Once dephosphorylated by the membrane-bound alkaline phosphatase (ALP), AMF is activated to a free thiol form (WR-1065), which is preferentially up taken by normal cells, since ALP is more active and efficiently expressed in normal rather than neoplastic tissue [46]. Moreover, in another study found that WR1065, the active free thiol form of amifostine, induces antioxidative ability against radiation via SOD2 *in vitro* [47,48]. Other studies have been shown the role of SOD2 in amifostine-induced protective effects, SOD2 mediated amifostine-induced antioxidative actions in PC12 cells exposed to glutamate. As SOD2 protein is mainly expressed in mitochondria which have been identified as a major source of ROS, we infer that high level of SOD2 protein may protect mitochondria by consuming ROS generated in oxidative injury. In addition, SOD2 mediated amifostine-induced effects on intracellular ROS, CAT, and GSH levels, indicating SOD2 may be the key target of amifostine in maintaining the balance of intracellular oxidants and antioxidants in PC12 cells(34). In our investigation we quantified the DNA-damage level, to elucidate the possible anti-genotoxic mechanism of amifostine against DOX-induced toxicity in $HepG_2$ cell line. Our results showed that DOX alone caused a significant increase in DNA fragmentation as compared to the untreated cells. However, treatment of HepG2 cells with amifostine 24h before DOX administration induced a noticeable decrease in DNA fragmentation as compared to the DOX-treated group. Measurement of ROS generation showed that DOX induced ROS generation. Amifostine is a potent cytoprotective agent that can inhibit oxidative stress by scavenging ROS and replenishing GSH.

Conclusion

In conclusion, we have demonstrated that amifostine protected Hepg2 cells against DOX-induced DNA damage and oxidative injury. Furthermore, we showed that DOX increased intracellular ROS generation and decreased intracellular GSH levels. Amifostine ameliorated the balance of intracellular antioxidants and oxidants, decreased ROS generation and enhanced the intracellular level of GSH.

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