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Research Article

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Genoprotective Effects of Amifostine against Mitomycin C-induced Toxicity in Human Hepatoma Cells

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Abstract

Mitomycin C is an antitumor antibiotic effective against many human malignancies. Numerous mechanisms have been offered for the anticancer effects of mitomycin C, such as DNA synthesis inhibition, DNA crosslinking and free radical generation. Amifostine is a cytoprotective agent used in cancer chemoprotection, implicating DNA-binding chemotherapeutic agents. The purpose of this study was to discover whether amifostine protects against mitomycin C -induced genotoxicity in HepG2 cells. Furthermore, we measured the DNA damage level with comet assay in HepG2 cells treated with mitomycin C and amifostine in different condition. Besides, we measured the intracellular ROS generation level and GSH levels in cells treated with mitomycin C and amifostine in pre-treatment condition. Our results presented that mitomycin C induced a concernable genotoxic effect in HepG2 cells. Amifostine attenuated the effects of mitomycin C significantly ($p<0.0001$) by reduction of the level of DNA damage via blocking ROS generation, and improvement of intracellular glutathione levels.

Keywords: Mitomycin c, Amifostine, Comet assay, ROS, Genotoxicity

Introduction

Mitomycin C (MMC) is a quinone-containing antibiotic initially isolated from *Streptomyces caespitosus* [1,2]. MMC has been used to treat an extensive types of cancers [3]. Although recent use of MMC is limited, this drug continues to be an important agent in numerous clinical trials because of its inherent effectiveness against many solid tumors and special activity in hypoxic tumoral cells [4]. MMC has a synergistic effect with radiotherapy through its radio sensitizing effects and targeting hypoxic cells in radiation resistant tumors [5]. As a potential alkylating agent, MMC needs a bioreductive transformation to form active species that crosslink DNA [6]. Due to the biotransformation route, metabolism of MMC may produce ROS. When ROS cooperate with cells and exceed endogenous antioxidant systems, there is unselective damage to biological macromolecules such as nucleic acids, proteins, and lipids [7,8]. Therefore, a comprehensive evaluation aimed to its side effects, such as genotoxicity which results in secondary malignancy is necessitated.

Amifostine is a phosphorothioate proposed as a radiation-protective agent used in cancer chemotherapy as a cytoprotective agent [9]. As stated by the diverse reports, inside the cell, amifostine's protective effects appear to be facilitated by scavenging free radicals, hydrogen donation, induction of cellular hypoxia and formation of mixed disulphides to protect normal cells [10,11]. Amifostine has shown notable radio- and chemoprotective effects in numerous studies. It is recently accepted for clinical use as a protective agent against renal toxicity result from cisplatin therapy in patients being treated for ovarian cancer and against xerostomia induced by ionizing radiation in patients with head and neck cancer [12-15]. In addition many studies has been showed that amifostine attenuate cardiotoxicity, nephrotoxicity and genotoxicity result from chemotherapy agents [13,19-21].

The single cell gel electrophoresis assay (SCGE, also known as comet assay) is an uncomplicated and sensitive technique for the detection of DNA damage at the level of the individual eukaryotic cell. Applications of this test include genotoxicity testing, human biomonitoring and molecular epidemiology, ecogenotoxicology, as well as primary research in DNA damage and repair [22-24]. The aim of present study was to explore the protective effect amifostine against mitomycin c- induced genotoxicity. So, we measured the DNA damage level with comet assay in HepG2 cells treated with Mitomycin c and amifostine in co and pre-treatment conditions. We also examined the generation of ROS and intracellular glutathione levels.

Materials and Methods

Chemicals

Mitomycin c 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 [25-28].

Cell culture

HepG2 cells were grown as monolayer culture in RPMI 1640 medium supplemented with 10% FBS, 1% of mixture of penicillin (100 IU/ml) and streptomycin (100µg/ml) incubated at 37 °C in an atmosphere of 5% CO_2–95% air mixture. Untreated cells closed as a control. Cells were cultured 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) 24 h prior and Simultaneously to mitomycin c treatment (0.5µg/ml) for 1 h at 37 °C [23].

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

The comet assay procedure has been described in our previous studies [29-32]. In brief, 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 min and rinsed with distilled water to remove the excess lysis solution. Then, slides were incubated with electrophoresis buffer (pH> 13.0) for 40 min. Electrophoresis was performed for 40 min at 25 V with an electricity current adjusted to 300 mA. After this stage, the slides were rinsed and were placed in the neutralization solution (pH=7.5) for 10 min. 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 [23]. All steps of comet assay were performed in dark conditions and all solutions were prepared freshly and used cool [25].

Measurement of Oxidative Stress

Approximately 4×10^4 cells per well were cultured for 24 h 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) 24 h prior Mitomycin c treatment (0.5µg/ml) for 1 h 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 10µM 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 H2O2 as a positive control [23].

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 $CaCl_2$, 0.5m $MMgSO_4$, 5 mg/ml BSA) for 30 min at 37°C in the dark. Although mBCI is a nonfluorescent 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 Mitomycin c (0.5µg/ml) in pretreatment condition(23).

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 mitomycin c -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 mitomycin c and prevented by amifostine were shown in Table 1. We observed that mitomycin c treatment at 0.5µg/ml 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 control group.

Table 1: The genoprotective 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. The sign (*) show significantly ($p<0.0001$) decreased compared to the mitomycin c group. (one-way ANOVA followed by tukeys post hoc test).

	Treatment	Tail length (Pixels) (Mean±SEM)	% DNA in Tail (Mean±SEM)	Tail moment (Mean±SEM)
Pre-treatment	Control (MMC 0.5µg/ml)	123.2±2.1	60. 5±1.9	42.3±1.45
	Amifostine (1mg/ml)	74.15±2.1	54.2±1.7	35.3±1.7
	Amifostine (5mg/ml)	26.12±0.9 *	10.2±0.7*	9.1±1.1*
	Amifostine (10mg/ml)	15.2±1.18*#	4.9±0.1*	1.1±.07*
Co-treatment	Control (MMC 0.5µg/ml)	123.2±2.1	60. 5±1.9	42.3±1.45
	Amifostine (1mg/ml)	83.3±1.5	60.17±1.6	39.40±1.4
	Amifostine (5mg/ml)	29.3±1.2*	14.3±0.44*	10.7±1.3*
	Amifostine (10mg/ml)	18.33±1.5*	5.3±0.2 *	1.2±.04*

Study the effect of amifostine on ROS generation in mitomycin c treated cells

To investigate the role of oxidative stress in mitomycin c -induced genotoxicity, we used DCFH-DA, a cell-permeable fluorescent dye, to examine the ROS generation in HepG2 cells in response to mitomycin c stimulation. Incubation with mitomycin c for 1 h showed a considerable increase in oxidant-induced 2_, 7_-dichlorofluorescein fluorescence in HepG2 cells (Fig. 1). H2O2-mediated DCF fluorescence occurred after 1h incubation with mitomycin c (0.5µg/ml) in HepG2 cells. This suggests that mitomycin c, 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 mitomycin c group. Untreated cells served as control.

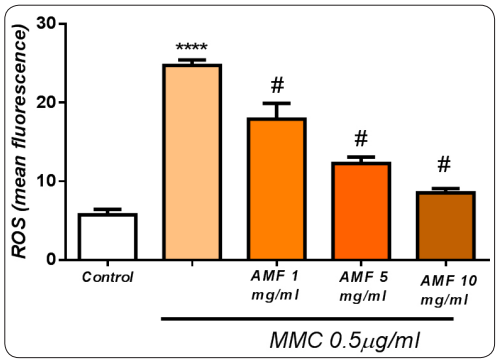

Figure 1: Study the effect of amifostine on mitomycin c -induced ROS generation. (****) show significantly increased results (respectively $p<0.0001$) as compared to the control group. The sign (#) show significantly ($p<0.0001$) decreased compared to the mitomycin c group.

Study the effect of amifostine on intracellular levels of GSH

We first examined the effect of mitomycin c 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 fig.2, within 1h after mitomycin c (0.5µg/ml) 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 mitomycin c in pre- treatment condition. As shown in fig.2 amifostine were significantly ($p<0.0001$) increased GSH levels as compared to the mitomycin c group.

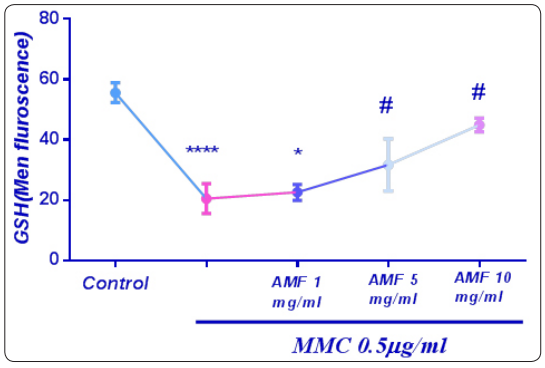

Figure 2: The effect of amifostine on the levels of intracellular GSH were determined. ANOVA analysis revealed that amifostine, significantly inhibited the effects of mitomycin c on the levels of GSH. Sign (****) and (*) show significantly decreased results (respectively $p<0.0001$and $p<0.05$) as compared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

Discussion

Chemotherapy-related unfavorable effects avert cancer patients from receiving ideal treatment and may obscure patient prognosis [26]. For the last decade, considerable development has been observed in the prevention and treatment of therapy-related problems. In cancer treatment, mitomycin c is a normally used drug against several human malignancies such as gastro-intestinal cancers, anal cancers, and breast cancers, as well as bladder tumors [27-28]. Genotoxic drugs affect both normal and cancer cells, but the selectivity associated with sensitivity of rapidly dividing cells such as cancer cells [29].

Amifostine has been currently accessible for chemotherapy- and radiation-related non hematological toxicity. Amifostine is a cytoprotective agent acting as a scavenger of free radicals, the most effective radio protector known and the only one accepted for clinical use in cancer radiotherapy [30]. It is a phosphorylated pro-drug, speedily dephosphorylated by alkaline phosphatase or mixed disulfides with endogenous thiols and thiol-containing proteins [17]. 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 [31]. Moreover, in another study found that WR1065, the active free thiol form of amifostine, induces antioxidative ability against radiation via SOD2 in vitro [32]. Moreover, WR-1065, produces cytoprotective effects by binding to and detoxifying directly the active forms of chemo cytotoxic drugs, scavenging free radicals, and donating hydrogen ions for DNA repair. The drug concentrates about 50- to 100-fold more in normal cells than in the tumor tissue; therefore, administration of amifostine results in cytoprotection without loss of any antitumor activity [33]. In experimental animals, Yuhas and Storer showed that treatment with AMF effectively protects normal tissue from the toxicity of therapeutic radiation, without protecting tumor [34]. 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 [35].

In our investigation we quantified the DNA-damage level, to elucidate the possible anti-genotoxic mechanism of amifostine against mitomycin c -induced toxicity in HepG2 cells. Our results showed that mitomycin c alone caused a significant increase in DNA fragmentation as compared to the untreated cells. However, treatment of HepG2 cells with amifostine 24 h before mitomycin c administration induced a noticeable decrease in DNA fragmentation as compared to the mitomycin c -treated group. Measurement of ROS generation showed that mitomycin c 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 mitomycin c -induced DNA damage and oxidative injury. Furthermore, we showed that mitomycin c 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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