

Evaluation of protective effect of amifostine on dacarbazine induced genotoxicity

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Abstract

Anticancer therapy with alkylating agents has been used for many years. Dacarbazine (DTIC) as an alkylating agent is used alone or in combination with other chemotherapy drugs. In order to inhibit the formation of secondary cancers resulting from chemotherapy with DTIC, preventional strategies is necessary. The present study was undertaken to evaluate the genoprotective effect of amifostine on the genotoxic effects of DTIC in cell culture condition. To determine the optimum genotoxic concentration of DTIC, HepG2 cells were incubated with various DTIC concentrations including 5, 10 and 20 $\mu\text{g/ml}$ for 2 h and the genotoxic effects were evaluated by the comet assay. The result of this part of the study showed that incubation of HepG2 cells with DTIC at 5 $\mu\text{g/ml}$ was sufficient to produce genotoxic effect. In order to determine the protective effects of amifostine on genotoxicity induced by DTIC, HepG2 cells were incubated with different concentrations of amifostine (2, 3 and 5 mg/ml) for 1 h which was followed by incubation with DTIC at 5 $\mu\text{g/ml}$ for 2 h. One hour incubation of cells with different concentrations of amifostine before incubation with DTIC indicated that at least 5 mg/ml concentration of amifostine can prevent genotoxic effects induced by DTIC on HepG2 cells under described condition. In conclusion amifostine could prevent DNA damage induced by DTIC on HepG2 cells.

Keywords: DNA damage; Comet assay; Dacarbazine; Amifostine

INTRODUCTION

Dacarbazine (DTIC) as an anticancer drug is used in combination with other chemotherapy drugs in the treatment of several cancer types such as Hodgkin's disease, malignant melanoma, soft tissue sarcoma, neuroblastoma and fibrosarcomas (1-7). Although the clear mechanism of action of this drug is not known, it seems to act as an alkylating agent (8-11). Liver has the key role in transformation of this prodrug to its reactive compound, methyl triazeno imidazole carboxamide (MTIC) which is able to attach an alkyl group to DNA. The repairing mechanisms of DNA could repair these kinds of defects by a repairing enzyme called O-6-methylguanine methyltransferase (MGMT). In the absence of active enzyme in repairing process, mutation which could be fatal to cells may occur (9). Several studies have shown that

DTIC could act as a purine analog in order to interact with sulfhydryl groups in inhibition of DNA, RNA and protein synthesis (9,11). Distribution of this drug to different parts of the body, could affect normal cells and as a result numerous side effects such as nausea, vomiting, neutropenia, myelosuppression and alopecia could take place. Chemoprotective agents and symptomatic treatments are suggested to reduce these side effects. Development of secondary neoplasia as a result of chemotherapy especially with alkylating agents is common (12-15). Collins and coworkers reported an acute myeloid leukemia as a secondary cancer following treatment by DTIC (16). Amifostine, an organic thiophosphate, could protect normal cells against toxic effects of anticancer drugs and radiotherapy, while it's not effective on neoplastic cells. Amifostine as a prodrug is activated by membrane-bound alkaline

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phosphatase to its active metabolite WR-1065 (17-19) acting as a scavenger of oxygen free radicals which is able to bind to platinum and alkylating agents (20). Higher concentrations of alkaline phosphatase in normal cells and higher pH of normal tissues in comparison with cancerous cells lead to the selective uptake of WR-1065 by normal cells (19,21,22).

Several methods have been applied to evaluate the DNA damages (23,24). Comet assay, known as Single Cell Gel method (SCG) has been introduced as a micro electrophoresis method for direct observation of DNA damages. The mechanism by which comet assay detects DNA damage has been explained previously (25). The cells trapped in the agarose gel are lysed under the alkaline pH to release DNA from the cells. Under the effect of electrical flow in electrophoresis, the DNA molecules move toward anode to form the comets. The comet formation pattern is determined by the size of the DNA fragments and the number of broken ends (26). As the extent of the damage increases, the free DNA fragments contain longer tails. To perform this test, a suspension of the separated cells should be prepared. DNA damage should be assessed in the cells without giving them the opportunity to be exposed to any other genotoxic agents (27). Microscopic observation of DNA migration is possible using ethidium bromide staining and a fluorescent microscope (28).

According to the wide application of DTIC in cancer treatment protocols and its serious side effects especially secondary cancers, seeking new strategies to prevent the side effects seems imperative. With regard to the preventative effects of amifostine on normal cells, this study was performed to evaluate the genoprotective and dose dependent effects of this drug on genotoxicity of DTIC on the metabolically competent human hepatoma cell line (HepG2 cells).

MATERIALS AND METHODS

Materials

DTIC and amifostine were respectively purchased from Medac Co. (Germany) and

MedImmune Pharma BV. (Poland). Tris, Triton X-100, H₂O₂, NaCl, ethylenediaminetetraacetic acid (EDTA), NaOH and NaH₂PO₄ were procured from Merck Co. (Germany). Low melting point agarose (LMA), Na₂HPO₄, KCl and ethidium bromide obtained from Sigma Co. (USA). Normal melting point agarose (NMA) was supplied by Cinnagen Co. (Iran), RPMI-1640, FBS and antibiotic were purchased from PAA Co. (Australia). HepG2 cells obtained from Pasture Institute (Iran).

Cell culture

HepG2 cell line was cultured in RPMI medium (containing 10 % fetal bovine serum and 250 µl of penicillin/streptomycin to avoid the growth of undesirable and pathogenic bacterial microorganisms) and incubated under 5 percent CO₂ at 37 °C in micro-filter plates. Cells were incubated with different concentrations of DTIC (5, 10 and 20 µg/ml) for 2 h to select the lowest genotoxic concentration of DTIC. In the next step HepG2 cell were incubated with the adopted DTIC concentration (5 µg/ml) for 2 h which was followed for further 1 h incubation in the presence of different concentrations of amifostine (2, 3 and 5 mg/ml). As described in our previous studies, the upper medium of each well was thrown away and wells were washed with PBS. Cells was dissociated from the culture surface using trypsin solution and then suspended in 1 ml fresh medium for the next stages of the comet assay (29,30).

Alkaline comet assay

The comet assay procedure has been described in our previous studies (29-32). Briefly, incubated cell suspensions (1×10^6 cells/ml) were mixed with 1% LMP agarose at 37 °C, 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 excess lysis solution. In the next step, slides were incubated with electrophoresis buffer (pH> 13.0) for 40 min. 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 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 barrier filter of 590 nm (23). All stages of comet assay were performed in dark conditions and all solutions were prepared freshly and used cool (29,30).

Statistical analysis

Tail moment (percentage of DNA in tail × tail length), tail length (the length of the comet tail), and percent of DNA in 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 (33,34).

One-way analysis of variance (ANOVA) followed by Tukey's multiple comparison post hoc test was used to compare the results of the comet assay.

The *p*-values of 0.05 and less were considered as statistically significant.

RESULTS

Study of genotoxic effects of dacarbazine

The genotoxic effect of DTIC was tested on the basis of the previous studies (6,35,36). To determine the most appropriate genotoxic concentration of DTIC, HepG2 cells were incubated with 5, 10 and 20 µg/ml of DTIC for 2 h before starting the comet assay (Fig. 1). One-way ANOVA for the results of tail length showed that tail length was increased significantly ($P < 0.0001$). Tukey's multiple comparison post hoc test indicated that the tail length was significantly ($P < 0.001$) increased at all concentrations of DTIC examined as compared to negative control group (Fig. 1A). One-way ANOVA followed by Tukey's multiple comparison post hoc test indicated that at all concentrations of DTIC the percentage of DNA in the tail was significantly increased ($P < 0.001$) compared to the control group (Fig. 1B). Statistical analysis also showed that the tail moment for all groups was increased significantly ($P < 0.0001$) (Fig. 1C).

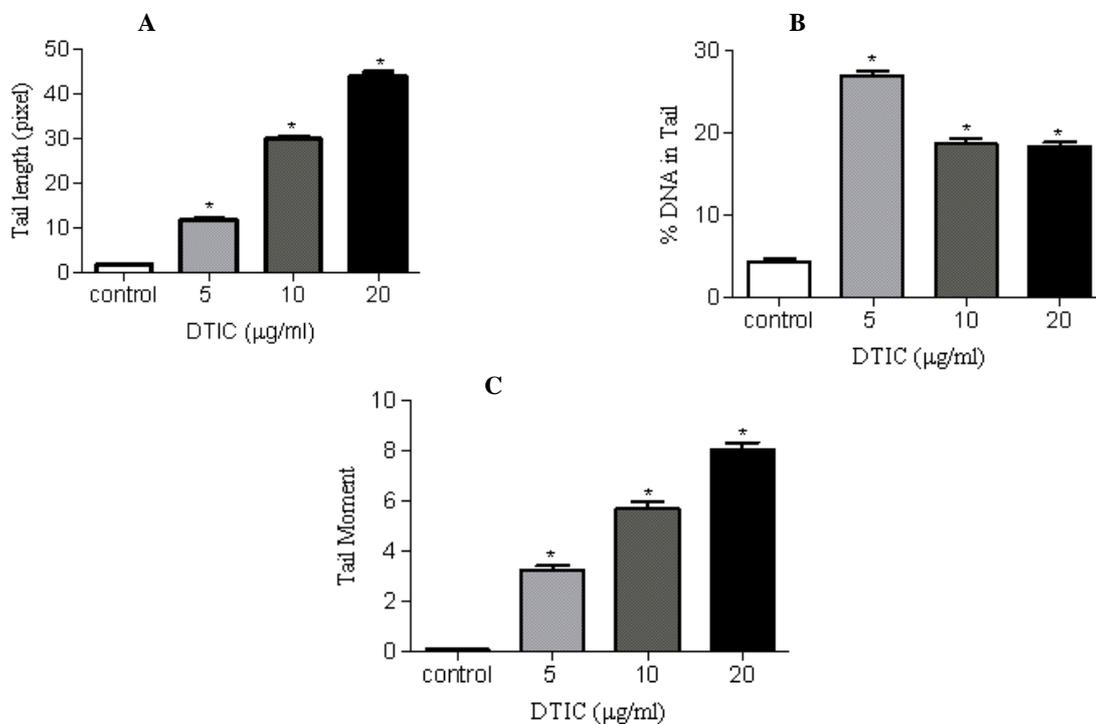


Fig. 1. Comparison of three studied factors in DTIC treated groups A; Tail length, B; % DNA in tail and C; Tail moment. Each graph has been represented as Mean ± SEM. The sign (*) shows significantly increased results ($P < 0.001$) in compare with the control group.

Up to this point, the studies indicated that all studied factors including tail length, percent DNA in the tail, and tail moment were increased at lowest DTIC concentration (5 $\mu\text{g/ml}$) amongst DTIC concentrations studied. Thus this concentration was selected for induction of the DNA damage for assessing the protective effects of amifostine.

Study of genoprotective effects of amifostine

In order to determine the protective effects of amifostine on genotoxicity induced by DTIC, HepG2 cells were incubated with different concentrations of amifostine (2, 3 and 5 mg/ml) for 1 h followed by incubation with DTIC at 5 $\mu\text{g/ml}$ for 2 additional h (Fig. 2). The ANOVA followed by Tukey's multiple comparison post hoc test showed that all

amifostine concentrations which was then accompanied by DTIC (5 $\mu\text{g/ml}$) were able to inhibit the genotoxic effects of DTIC and decreased the tail length significantly ($P<0.001$) concentration dependently as compared with the DTIC group (Fig. 2A).

The percentage of DNA in the tail and the tail moment ($P<0.001$) decreased significantly at 3 and 5 mg/ml of amifostine, while at the concentration of 2 mg/ml of amifostine the percentage of DNA in the tail decreased less proportionally than other two concentrations ($P<0.05$) as compared with the DTIC group (Fig. 2B and 2C).

Different concentrations of amifostine (2, 3 and 5 mg/ml) tested by comet assay method after 1 h incubation did not show genotoxic effect on HepG2 cells.

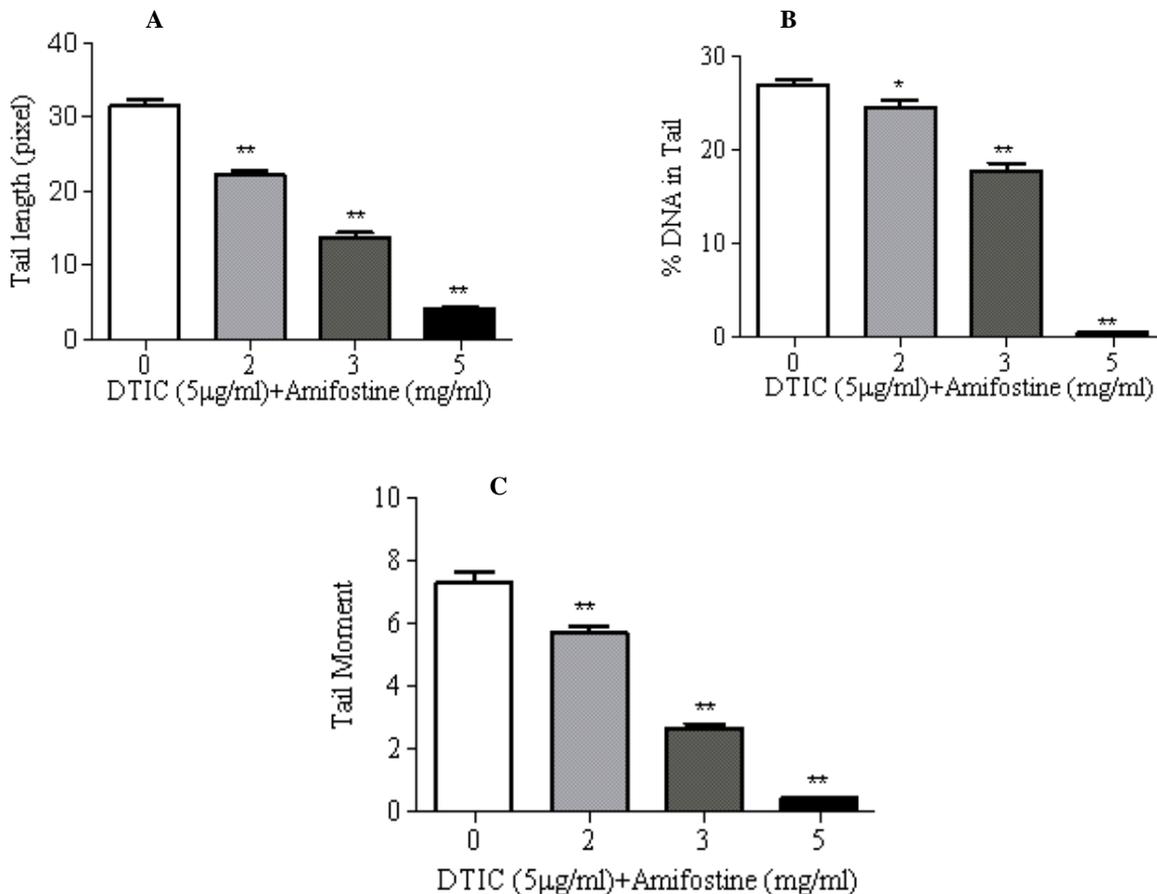


Fig. 2. Comparison of three studied factors in amifostine plus DTIC treated groups A; Tail length, B; % DNA in tail and C; Tail moment. Each graph has been represented as Mean \pm SEM. The sign (**) and (*) show significantly decreased results (respectively $P<0.001$ and $P<0.05$) in compare with the DTIC group.

DISCUSSION

The results of this study indicated that concentrations of 5, 10 and 20 µg/ml of DTIC are genotoxic on HepG2 cells after incubation for 2 h. The concentration 5 µg/ml was recognized as the lowest concentration of DTIC being genotoxic on HepG2 cells under the condition already described. All three concentrations of amifostine (2, 3 and 5 mg/ml) were found to protect the genotoxic effects of DTIC on HepG2 cells. Alkylating agents such as DTIC are used as a chemotherapeutic agent in the treatment of various cancers (37-39). The most important side effect of these classes of drugs is apparition of secondary neoplasia or cancers in additional sites (12-15). Alkylating agents substitute alkyl groups on the DNA leading to the damage of DNA, breaking labile bonds of DNA, formation of micronucleus, and finally leading to chromosomal breaking and genome instability.

These damages lead to the inhibition of biosynthesis pathways, cell cycle arrest, teratogenicity and apoptosis (35,40). DTIC may inhibit DNA and RNA synthesis by acting as a purine analogue. This drug is biotransformed to MTIC by demethylation in the liver and then to diazomethane, which attacks to the nucleophilic groups on DNA (8,35,40,41). Amifostine is approved by FDA to be used for reducing the side effects of cisplatin in patients with advanced ovarian cancer (42). Nowadays, amifostine known as a selective cytoprotective agent of normal tissues against the toxicity of chemotherapy and radiotherapy which acts as a scavenger of free radicals and conjugate of electrophilic substances (17, 43-46). WR-2721, a prodrug, which is dephosphorylated by *alkaline phosphatase* can activate free thiol metabolites in the tissues. The selective protection of non-malignant tissues is believed to be due to the higher *alkaline phosphatase* activity in normal tissues, different mechanisms of amifostine uptake, and different membrane-bound alkaline phosphatase concentrations (40,41, 47-50). The genotoxic effects of doxorubicin after 3, 6 and 9 h exposure to 10 µg/ml doxorubicin have been previously reported.

The results of this study showed time dependent genotoxicity of doxorubicin (51). Buschini and coworkers determined cytoprotective effect of amifostine (0, 50 and 100 µg/ml) on bleomycin genotoxicity by the comet assay. In this study, amifostine could reduce bleomycin genotoxic effects (52). In another study, amifostine was shown to have selective protection against melphalan-induced DNA damage in normal and tumoral cells (53). Amifostine was also proven to be an effective cytoprotector against the toxic effects of cisplatin (54). Blasiak and coworkers evaluated cytoprotective effects of vitamin C and E and amifostine on idarubicin genotoxicity on lymphocyte cells. Vitamin C and amifostine (14 mM) reduced DNA damage induced by idarubicin while vitamin E increased DNA damage of idarubicin (55).

CONCLUSION

The result of our investigation showed that lowest concentration of DTIC (5 µg/ml) could be genotoxic HepG2 cells incubated for 2 h. One-hour incubation of cells with different concentrations of amifostine before incubation with DTIC (5 µg/ml) indicated that studied concentrations of amifostine are able to prevent genotoxic effects of DTIC on HepG2 cells. It can be concluded that amifostine could prevent genotoxic effect of DTIC on HepG2 cells and could be suggested to be included in the chemotherapy protocols containing DTIC in order to prevent formation of secondary cancers.

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