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Assessment of radioprotective effects of amifostine on human lymphocytes irradiated *in vitro* by gamma-rays using cytokinesis-blocked micronucleus assay

H. Mozdarani[1*], A. Taheri[2], S.A. Haeri[2]

[1] Department of Medical Genetics, School of Medical Sciences, Tarbiat Modares University, Tehran, Iran
[2] Novin Medical Radiation Institute, Tehran, Iran

Background: A radioprotective effect of amifostine as well as its ability to modulate the level of spontaneous and gamma-rays-induced genetic changes on human peripheral blood lymphocytes has been investigated. Amifostine, known as a potent radical scavenger, has been introduced as the most effective radioprotector, yet it is not completely approved for the clinical use. However, further *in vitro* and clinical studies are needed to clarify its mechanisms of action. ***Materials and Methods:*** Whole blood samples from healthy donors were exposed to various doses of gamma-rays. Lymphocytes in cultures were treated with amifostine at different concentrations (2, 4 and 6 mM) in the presence or in the absence of 1 U/ml alkaline phosphatase before or after gamma-irradiation. Standard procedure for the cytokinesis-block micronucleus (CBMN) assay was used to assess the effect of amifostine on radiation induced micronucleus in binucleate lymphocytes. ***Results:*** Irradiated blood samples showed an increase in the total number of micronuclei (MN) significantly different from controls ($p<0.05$). However, pre-treatment of lymphocytes with amifostine in the presence of alkaline phosphatase, 15 minutes before irradiation, led to a significant decrease in the frequencies of MN and cells with more than one MN ($p<0.05$). Amifostine, in its own, produced little or no protection. However, the addition of amifostine with alkaline phosphatase to the cell cultures 15 minutes after irradiation produced substantial radioprotection significantly different from the frequencies of MN induced by radiation alone ($p<0.05$). ***Conclusion:*** Results clearly indicated that gamma-rays induced MN in lymphocytes in a dose dependent manner. The highest protective effect was achieved when amifostine was phosphorilated by alkaline phosphatase and present before irradiation in the cellular environment, was indicating its radical scavenging mechanism of radioprotection. Since the administration of amifostime after irradiation also led to a considerable decrease in the frequency of radiation induced MN, other mechanisms such as induction of cell cycle delay and hence influencing DNA repair, might be involved in radioprotection by amifostine. **Iran. J. Radiat. Res., 2007; 5 (1): 9-16**

Keywords: *Amifostine, gamma-rays, radioprotection, human lymphocyte, micronuclei.*

INTRODUCTION

Wide varieties of people are exposed to ionizing radiation and are potentially at an increased risk for adverse health effects. Included are victims of nuclear fallout, victims of nuclear terrorism, workers in the nuclear power industry, waste clean-up crews, people living in homes surrounding nuclear plants or research laboratories with radiological facilities, patients undergoing routine diagnostic or therapeutic radiation treatment procedures, astronauts occupationally exposed to cosmic radiation and members of the armed forces potentially subjected to intentional sources of radiation. An efficient radioprotector could prove to be useful in occupational and therapeutic settings, where ionizing radiation is used, or where exposure occurs, after nuclear accidents which leave radioactivity in the environment, and during space travel, to protect astronauts from the effects of high doses of radiation associated with solar flares [(1)].

The use of chemical agents to provide protection against radiation injury has been a major field of study for more than 5 decades. The discovery of the radioprotective effects of cysteine in rats and mice by Patt *et al.* (1949) [(2)] paved the way for researches on radiation protection in human. Since then,

Corresponding author:
Dr. Hossein Mozdarani, Dept. of Medical Genetics, School of Medical Sciences, Tarbiat Modares University, Tehran, Iran. P.O.Box: 14115-111
Fax: *+98 21 88006544*
E-mail: *mozdarah@modares.ac.ir*

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there has been an explosion in studies on radioprotection, and compounds with varied structures and physiological functions have been tested for their radioprotective abilities. However, these researches have not yielded a single compound which can be recommended for the use in human radioprotection. Phosphothioates are the most extensively studied, and the best normal tissue protectors available, so far. Amongst the phosphotioates, amifostine (synonyms: WR-2721, ethyol) was found to be the most effective compound to protect against damages caused by ionizing radiation [3]. Amifostine is an inactive prodrug that cannot protect cells until dephosphorylated to the active metabolite, WR-1065, by alkaline phosphatase in the plasma membrane [4]. The WR-1065 has shown remarkable radio- and chemoprotective effects *in vitro* and *in vivo* and 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 [5]. Preclinical studies have shown that administration of WR-2721 before irradiation protected against radiation clastogenesis [6, 7], mutagenesis and carcinogenesis [8].

Ionizing radiation forms radicals in the DNA (direct effect) and in the surrounding water molecules of the hydration shell of the DNA (indirect effect), which in turn destroy DNA [9]. Since radiation-induced cellular damage is attributed primarily to the harmful effects of free radicals, molecules with radical scavenging properties are particularly promising as radioprotectors [10, 11].

The protection of cell damage by WR - 1065 is thought to occur through scavenging oxygen derived free radicals (induced by ionizing radiation and certain types of chemotherapy), to participate in direct chemical repair of damaged target molecules through the donation of hydrogen atoms [12] and to induce intracellular hypoxia as a result of undergoing auto-oxidation [13]. Each of these mechanisms requires that WR-1065 must be present at the time of radiation or drug treatment [14]. Due to the need to phosphorylation and formation of active metabolite WR-1065, relatively few *in vitro* studies have been done by amifostine, i.e. most of the *in vitro* studies were performed using WR-1065. Recent reports describing the combined effects of amifostine and melatonin on gamma rays induced micronuclei *in vitro* [15] highlights the need for further *in vitro* studies to elucidate the anti-clastogenic effects and mechanism of amifostine.

DNA damage induced by gamma-rays in the presence of amifostine was estimated using well-established biomarker, the cytokinesis-block micronucleus assay (CBMN) [16, 17]. DNA damages induced by ionizing radiation, mainly strand breaks, converts to structural chromosomal aberrations. A proportion of the aberrations (usually referred as "asymmetrical events" or "unstable aberrations") [18] give rise to chromosome fragments or "acentric fragments" (AF) without spindle attachment organelles (kinetochores, centromeres). After exposure to genotoxic agents such as ionizing radiation, micronuclei (MN) in the cytoplasm of interphase cells are either derived from acentric chromosomal fragments or from whole chromosomes that lag behind in anaphase, and they are not included in the daughter nuclei in telophase as small, extranuclear bodies [19-21]. By adding cytochalasin B to the lymphocyte cultures cytokinesis is blocked without inhibiting nuclear division. Cytokinesis-blocked cells accumulate in the firstly division cycle and can be easily identified from their binucleate appearance. Scoring of MN in cytokinesis blocked binucleate cells has been suggested as a sensitive method to assess cytogenetic response of cells to ionizing radiation [22].

The aim of this study was to examine genotoxic and radioprotective effects of amifostine in the presence or in the absence of alkaline phosphatase, administered before and after gamma-irradiation of human lymphocytes by means of cytochalasin B

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blocked micronucleus assay.

MATERIALS AND METHODS

Amifostine treatment and gamma irradiation

Whole blood samples were collected from two non-smoking healthy male volunteers (mean age 30 ± 2 years) who had no history of previous exposure to other clastogenic agents at least one month prior to sampling. Blood samples were set up for different treatments in microtubes. Amifostine (Schering-Plough, Netherlands) at various concentrations (2, 4 and 6 mM) was added to the cultures with or without alkaline phosphatase (1U/ml, Fluka). Amifostine treatment was done either 15 min before or 15 min after gamma-irradiation. Lymphocytes in whole blood cultures were irradiated with various doses of gamma rays (from 1.5-6 Gy) generated from a cobalt 60 source (Theratron II, 780 C, Canada) at a dose rate of 1.54 Gy/min with a source surface distance (SSD) of 80 cm and fixed field size of 10×10 cm^2 at room temperature (23 ± 2 °C). Radiation dose of 6 Gy was used for irradiation of amifostine treated samples.

In vitro micronucleus assay

CBMN was performed using a standard protocol, as described by Fenech (2000) [20]. Briefly, 0.5 ml of whole blood was cultured in 4.5 ml RPMI-1640 medium (Sigma) supplemented with 15% inactivated fetal calf serum (Gibco-BRL), antibiotics (Penicillin, 100 IU/ml and Streptomycin, 100 µg/ml), L-glutamine and 0.1 ml of phytohemaglutinin (PHA) (Gibco-BRL) at a final concentration of 5 µg/ml as mitogen to each culture vessel. 28 h after culture initiation, cytochalasin-B (6 µg/mL) was added to the cultures and cells were harvested at 72 h. Cells were exposed to hypotonic solution (KCl, 0.075 M) for 1 minute, and then fixed in Carnoy's fixative (6:1 v/v methanol: glacial acetic acid, Merck). Slides were prepared using air drying technique and stained in 5% Giemsa solution (Merck). Cells were scored according to the criteria outlined by Fenech *et al.* (2003) [21]. The frequency of MN was determined by scoring 1000 binucleate lymphocytes per sample. Slides were analyzed blind at ×400 magnification under a light microscope (Nikon, Japan).

Statistical analysis

Non parametric Mann-Withney *U-test* and one way analysis of variance (ANOVA) was used for statistical analysis to determine whether there was any statistical difference in the frequency of micronuclei induced by gamma-irradiation in lymphocytes in the absence, or in the presence of amifostine. *P-value* of less than 0.05 was considered as a significant level.

RESULTS

Results are summarized in table 1 and shown in figures 1-3. The background level of MN in blood donors was 20 MN per 1000 binucleated cells (table 1). As seen, the frequency of MN increased dramatically after gamma-irradiation in a dose dependent manner (figure 1), so that at the dose of 6 Gy the number of MN in the exposed lymphocytes has increased over 22-folds (453 MN per 1000 binucleated cells). Treatment of lymphocytes with amifostine in the presence of alkaline phosphatase showed no or little genotoxicity over the dose range used in this study (2-6 mM). Although the frequencies of

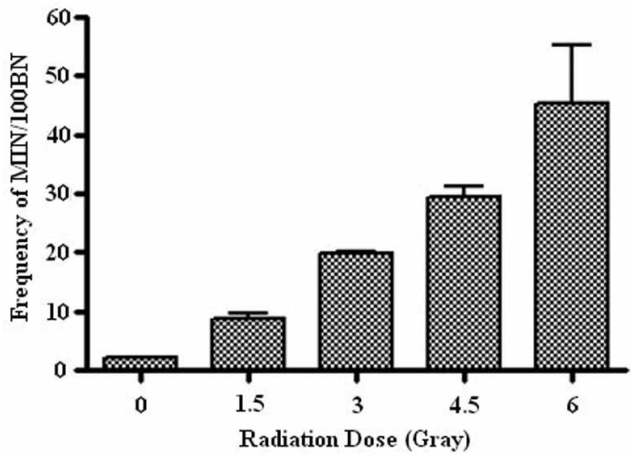


Figure 1. Frequency of MN in binucleate lymphocytes following irradiation with different doses of gamma-rays.

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Table 1. Frequency and distribution of micronuclei in binucleate human lymphocytes irradiated with gamma rays in the presence and in the absence of various concentrations of amifostine.

Treatment	Amifostine Concentration (mM)	No. Binuclei Scored	Distribution of micronuclei					Mean No. MN/1000 Binuclei cells
			0	1	2	3	4	
Control	0	2000	1962	36	2	0	0	20 ± 1.4
Amifostine + AP*								
	2	2000	1944	49	5	1	1	32.5 ± 16.2
	4	2000	1924	72	4	0	0	40 ± 0
	6	2000	1933	64	3	0	0	35 ± 1.4
Radiation only								
1.5 Gy	0	2000	1834	157	9	0	0	87.5 ± 13.4
3 Gy	0	2000	1643	317	38	2	0	199.5 ± 3.5
4.5 Gy	0	2000	1497	425	70	7	1	295.5 ± 23.3
6 Gy	0	4000	2655	955	309	50	21	453 ± 198.5
Amifostine + 6 Gy (-15 min)**								
	2	2000	1461	416	108	12	3	340 ± 5.6
	4	2000	1455	407	100	26	12	365.5 ± 153.4
	6	2000	1380	471	131	13	5	396 ± 107.4
Amifostine + AP + 6 Gy (-15 min)								
	2	4000	3243	602	144	9	2	231.5 ± 39.6
	4	4000	3174	666	143	15	2	250.7.5 ± 41.2
	6	4000	3062	753	165	17	3	287 ± 78.7
Amifostine + AP + 6 Gy (+15 min) #								
	2	2000	1577	347	70	5	1	253.5 ± 92.6
	4	2000	1462	445	87	5	1	319.5 ± 37.4
	6	2000	1425	446	110	16	3	363 ± 12.7

*AP = alkaline phosphatase; ** -15 min = administered 15 minutes before irradiation;
+15 min = administered 15 minutes after irradiation

MN induced by amifostine treatment was higher than control value, but the difference was statistically non-significant ($p>0.05$).

Addition of amifostine with different concentrations without alkaline phosphatase treatment, 15 minutes before 6 Gy gamma-irradiation, led to a slight reduction in the frequency of MN. This reduction in MN frequency was significantly different from the frequencies of radiation induced MN ($p<0.05$) only for 2 mM concentration. Slight or no protective effect was seen with higher doses of amifostine. However, protection of amifostine against 6 Gy gamma-rays induced MN was much greater with all concentrations when used with alkaline phosphatase and significantly different from the frequency of MN induced by radiation alone ($p<0.05$) (table 1 and figure 2). With concentrations of 2 and 4 mM amifostine, a dose reduction factor (DRF) of 2.1 was observed, but DRF for 6 mM concentration of amifostine was slightly lower at about 1.7. Figure 2 shows the difference in protecting

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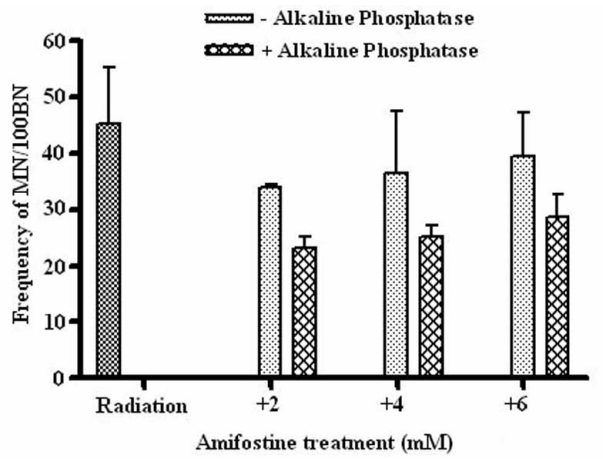


Figure 2. Comparison of the effects of various concentrations of amifostine on 6 Gy gamma-rays induced MN when used with or without alkaline phosphatase (1 IU/ml) treatment.

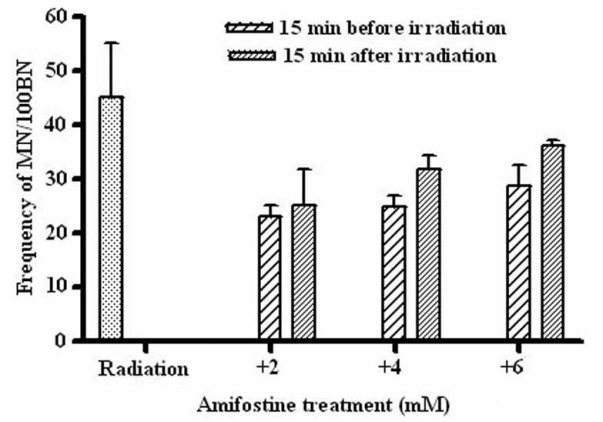


Figure 3. Comparison of the effects of amifostine with various concentrations on the frequencies of MN in binucleate lymphocytes when administered either 15 min before or 15 min after 6 Gy gamma-irradiation.

effects of amifostine in the presence and in the absence of 1 U/ml alkaline phosphatase. Frequencies of binucleate cells with more than 1 MN decreased in all amifostine treated samples (table 1).

Figure 3 shows the effects of amifostine in the presence of alkaline phosphatase on the frequency of radiation induced MN when administerd 15 minutes after irradiation of lymphocytes cultures. As seen, the frequency of radiation induced MN decreased with all concentrations of amifostine, significantly different from the frequency of MN induced by radiation alone ($p<0.05$). However, the protective effect was much lower in comparison with amifostine treatment 15 minutes before irradiation (figure 3).

DISCUSSION

Clastogenicity of various doses of gamma-rays used in this study is shown by the increase in the incidence of MN in exposed human lymphocytes in a dose dependent manner (table 1 and figure 1). This effect has been reported in many other *in vitro* and *in vivo* investigations previously [23-27]. High frequencies of MN were produced by the dose of 6 Gy gamma-rays. This dose of radiation was chosen to study the potential radiprotective effects of amifostine.

Studies with cultured cells demonstrate little or no protection when the cells are treated with phosphorothioate drugs. Results shown in table 1 and figure 2 clearly indicates that amifostine without being phosphorilated into active metabolites, has produced little or no protective effects against radiation induced MN. This observation was similar for all dose ranges used in this study (2-6 mM). Enzymatic action of purified alkaline phosphatase on WR-2721 is known to improve its *in vitro* radioprotection in tissue culture [28, 29]. The addition of only 1 U/ml of alkaline phosphatase led to a substantial decrease in the frequency of radiation induced MN (figure 2), indicative of phosphorylation of amifostine to active metabolite form of WR-1065. Previous studies demonstrated that WR-1065 protects cells against radiation-induced chromosome aberrations and micronuclei in G0 human lymphocytes irradiated with X-rays and neutrons [7, 14, 29-33].

It has been suggested that amifostine is radioprotector, reducing the toxicity and biological effects of ionizing radiation through the scavenging of hydroxyl radicals, transferring hydrogen to DNA radicals, and causing a hypoxic state near DNA [34]. It has been demonstrated that amifostine protects total-body irradiated mice against the toxicity induced by X-rays by inactivating the oxygen-derived free radicals formed during water radiolysis. In an *in vitro* model it has

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been shown that WR-1065 protects human premonocytic cell line U937 from H_2O_2-induced cell death in a dose-dependent manner, and more efficiently than WR-2721 [35].

It is known that the clastogenic effects of ionizing radiation are due to the formation of free radicals leading to production of DNA strand breaks initiating several cellular processes including cell killing, mutagenesis, transformation and carcinogenesis. It is reasonable to assume that the agents capable of scavenging free radicals would play a significant role in modulating these processes. Radical scavengers can efficiently protect the cells toward DNA strands breakage [36]. The major mechanism in protection by compounds such as WR-2721 is thought to be that of free radical scavenging. Reduction in the frequency of MN in amifostine treated cells with alkaline phospatase by a factor of about 2 may be due to this property of this agent (figure 2). It was shown that hydroxyl radical scavenging and DNA radical repair are two important mechanisms in the protection of cells by thiols and that the net charge on the thiol is a significant factor to its effectiveness [37]. WR-2721 has shown to inhibit Fenton-reaction generated free radicals *in vitro* [38]. Using supercoiled plasmid DNA and restriction fragments, Savoye *et al.* (1997) [39] have shown that under anaerobic conditions WR-1065 protects by scavenging of OH radicals and chemical repair by H donation. However, results shown in table 1 and figure 3 for the effect of amifostine on gamma-rays induced MN when administered 15 min after irradiation might suggest that such protection may reasonably be mediated by mechanisms other than free radical scavenging, hydrogen atom donation and / or induced oxygen depletion. In line with these observations, it was demonstrated that WR-1065 was able to reduce the frequency of radiation-induced mutations significantly, as a result of post-irradiation exposure [14, 33]. Therefore, it is likely that the aminothiols exert at least some of their radioprotective effects by influencing DNA repair [33, 40, 41] and intracellular release of glutathione [7]. These compounds are known to inhibit both DNA synthesis and cell cycle progression in exponentially growing cells in culture. WR-1065, in particular, perturbs cell cycle progression, resulting in accumulations of cells at S and G_2. Such delays may increase the time available for repair of DNA lesions before they are converted to irreversible mutations [33]. These findings are consistent with the hypothesis that the exposure of cells to WR-1065 results in catalytic inactivation of topoisomerase II. This topoisomerase II inactivation would have the effect of slowing cell cycling, thus providing more time for DNA repair to occur [42]. This could result in protection of normal tissues against the clastogenic effects of radiation. Such differential protection by WR-1065 is well documented in the case of gamma-irradiation.

In conclusion, data presented in this study clearly showed the positive effect of alkaline phosphatase on the radioprotective efficacy of amifostine. The highest protective effect was achieved when amifostine was phosphorilated and present before irradiation in the cellular environment, indicating its radical scavenging mechanism of radioprotection. Since the administration of amifostime after irradiation, also led to a considerable decrease in the frequency of radiation induced MN, it might be possible other mechanisms, such as induction of cell cycle delay and hence, influencing DNA repair, to be involved in radioprotection by amifostine.

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