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Evaluation of the role of mannitol in radioprotection of amifostine

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ABSTRACT

Background: Mannitol is present in the Ethyol® (the trade name of amifostine) as an excipient. The mechanism of radioprotection of amifostine is radical scavenging. Since mannitol is another known radical scavenger, we studied the probable additive or synergistic effect of mannitol on the effect of amifostine.

Material and Methods: Mice were irradiated with Co-60 γ-ray in the presence 400mg/kg mannitol alone or in combination with 400mg/kg amifostine. Survival of mice was assessed within 30 days after irradiation (LD 50/30). Moreover, the protective effect of drugs was evaluated using micronuclei (MN) assay. Slides were prepared using femoral bone marrow flush and stained in May-Giemsa. The frequency of MN was determined in polychromatic erythrocytes (PCE) for each sample.

Results: Similar LD50/30 was observed for irradiated mice in the presence of amifostine alone or in combinations with mannitol. High frequency of MN was produced by 3 Gy γ-rays. Amifostine reduced radiation induced MN dramatically, but mannitol had no effect on γ-rays induced MN. Combination of mannitol with amifostine did not change the effect of amifostine alone.

Conclusion: Radioprotection of ethyol is due to the effect of amifostine. Presence of mannitol apparently has no role in radioprotective effect of amifostine. *Iran. J. Radiat. Res., 2003; 1(3): 151 - 155*

Keywords: *Ionizing radiation, free radicals, mannitol, amifostine, micronuclei, LD50/30.*

INTRODUCTION

Ionizing radiation can damage macromolecules in living cells. These damages can cause cell death, mutations and cancers. Most important of these effects are various types of DNA damage as most important target molecule in the cell.

However, these effects can be induced directly or indirectly. Water as a most important molecule in living cells, changes to free radicals such as hydroxyl radical (OH°), hydrogen (H°) and also H2O2 after irradiation. These free radicals are very active and react with surrounding molecules such as DNA, proteins and cell membrane lipids.

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Various chemical agents have been used to decrease radiation side effects with different mechanisms (Conklin and Walker 1987). Of the most important radioprotective agents are free radical scavengers, because these reactive species are mainly produced by sparsely ionizing radiations. Of the free radical scavengers, aminothiols and amifostine are the most potent radioprotectors in therapeutic and research applications (Conklin and Walker 1987).

Mannitol is a hexahydric alcohol from mannose family that dissolves in water. This sugary substance that has hyperosmolar effect in concentration above 5.07% has been used as a edema-control agent in patients who suffer from edematous disorders.

This substance has diuretic effect and has been injected to patients with a dose about 50-200 gr/day. Treatment of renal failure, intracranial edema and lung edema with mannitol is a routine

therapeutic procedure. Mannitol has been used as a diluent and excipient in pharmacology (Martindale 2000). Moreover, mannitol as an alcoholic sugar has free radical scavenging effect. This substance has been showed protective effect against both chemicals and ionizing radiations (Panda *et al.* 1995, Tachon 1990, Eichler 1987).

Amifostine (Ethyol) is a well – known radio- and chemoprotective agent which is lyophilized in 500mg vials. Since mannitol is added to amifostine vials as an excipient, synergistic or additive effect of mannitol on radioprotective effect of amifostine is possible. In this study we evaluated the presence of mannitol on radioprotective efficiency of amifostine by the use of micronucleus assay as a standard method for genotoxic studies, and assessment of LD50/30 as a standard survival assay method.

MATERIALS AND METHODS

Animals: Female Balb/C mice with 23.9 ± 5 gr weight were purchased from Razi-Institute (Karaj, Tehran) and kept in animal house at temperature of 23±2°C with %40 ± 5 humidity and 12-hours dark-light cycle for two weeks. (five mice for micronuclei test and 10 mice for LD50/30 test was used for each treatment group). Mice were nourished with standard mouse pellet and potable water.

Drugs: Amifostine (pure substance) synthesized in Bioscience and Biotechnology Research Center prepared and dissolved in normal saline (N/S). Injectable mannitol and N/S prepared from Daru-Pakhsh Co. Amifostine and mannitol at a dose of 400mg/kg and N/S at 0.01ml/g body weight were injected i.p.

Radiation: Mice were irradiated with 1 and 3 Grays, at a dose rate of 0.95 Gy/min and 80 cm SSD (Source – Sample Distance) with ^{60}Co gamma rays using a radiotherapy unit (Theratron-780, Canada) in Novin Medical Radiation Institute.

Micronucleus assay: Mice were randomized in pentamerous groups and each group was injected with N/S, mannitol (400mg/kg) or amifostine (400mg/kg) and amifostine + mannitol by insulin syringe from i.p rout. 15 minutes after injection, radiation test groups were placed in plexiglass irradiation box and irradiated. Fourty-eight hours after irradiation all mice were sacrificed with cervical dislocation. Then both femurs of each mouse were excided; bone marrow was flushed gently with homogenized and centrifuged fetal bovine serum. Bone marrow suspension was dropped on a clean-cold slide and left in room temperature for 24 hours. Then slides were stained with May-Graunwald and Giemsa stains.

Frequency of micronuclei in 1000 PCE were scored for each sample. Also proliferation rate of bone marrow erythrocytes was assessed by scoring both polychromatophylic erythrocytes (PCEs) and normochromatophylic erythrocytes (NCEs).

***LD50/30 assay*:** Ninety-eight mice distributed randomized in 5 main groups including control, solvent (0.01 ml/g), mannitol (400mg/kg), pure amifostine (400mg/kg) and amifostine plus mannitol (each drug 400mg/kg). Treatment groups were divided in subgroups by increasing the radiation dose exponentially. All drugs were injected intraperitoneally 15 min before irradiation. The rate of mortality was observed for 30 days postexposure.

Statistics: Results of micronucleus and cytotoxicity study were analyzed with one-way nonparametric ANOVA (Kruskull-Wallis test). LD50 /30 was determined by "moving average interpretation method". This animal saving method was introduced in 1974 by Thompson and it's software prepared by Schaper in 1994 (Schaper *et al.* 1994).

RESULTS

Micronuclei study:

The results obtained with micronuclei assay is summarized in table 1. As seen , radiation has dramatically increased the frequency of MN in PCEs specially when the dose of 3 Gy was used. Amifostine and mannitol were not genotoxic when used alone because did not increase the frequency of MN above control level. Presence

of mannitol, before irradiation did not change the frequency of radiation induced MN significantly. For samples injected with amifostine 15 min before irradiation, a dramatic decrease in frequency of MN was observed ($P<0.05$).

Combination of mannitol with amifostine did not change the effect observed by amifostine alone. Use of amifostine and mannitol either alone or in combination could not modify radiation induced cytotoxicity in bone marrow (table 1).

Table 1. Frequency of micronuclei and PCE rate after γ-ray irradiation in the presence or absence of mannitol and amifostine or in combination.

Treatment	Micronuclei/1000 PCE (Mean ± SD)	%PCE (Mean ± SD)
N/S + 0Gy	3.3 ± 0.4485	49.126 ± 0.832
NS+1Gy	32.6 ± 6.189	22.492 ± 1.450
N/S+3Gy	193.0 ± 8.955	7.117 ± 0.2397
Amifostine+0Gy	6.1 ± 0.7667	41.528 ± 0.97
Amifostine+1Gy	11.2 ± 0.5121	26.855 ± 2.548
Amifostine+3Gy	62.7 ± 3.757	13.106 ± 0.747
Mannitol+ 0Gy	6.8 ± 0.3633	51.639 ± 6.591
Mannitol + 1Gy	29.6 ± 0.924	23.612 ± 4.337
Mannitol±3Gy	168.34 ± 18.429	6.109 ± 0.522
Mannitol+Amifostine+0Gy	3.356 ± 0.129	46.821 ± 8.096
Mannitol+Amifostine+1Gy	15.753 ± 2.298	24.361 ± 3.607
Mannitol+Amifostine+3Gy	56.547 ± 9.376	10.528 ± 1.672

Survival study:

Figure 1 shows the mortality rate of mice irradiated in the presence of amifostine (WR) and mannitol alone or in combination at a dose of 400mg/kg. As shown mannitol and N/S (as solvent) have nearly similar LD50/30, thus no different in mortality rate. Amifostine with or without mannitol also show the same results.

DISCUSSION

Mannitol as an alcoholic sugar has anti-oxidant effect. This agent decreases the genotoxicity and cytotoxicity of chemicals (Panda *et al*. 1995, Tachon 1990) and radiation (Eichler *et al.* 1987 , Peak *et al.* 1990). Mannitol exerts these effects with different mechanisms. Some experiments have shown that mannitol can prevent inactivation of enzymes such as G6PD, Nitrate- reductase and sulfite oxidase by ionizing radiations (Eichler *et al*. 1987). Mannitol can protect supercoiled DNA from hydroxyl radical damage due to ionizing radiation (Peak *et al.* 1990, DeSesso *et al.* 1990).

Mannitol as a free radical scavenger has been used repeatedly in experiments and compared with other free radical scavengers. The substance is considered as a strong free radical scavenger (Kamat *et al.* 2000, Sagone *et al.* 1983).

By addition of mannitol in culture media of malignant cells, radiation induced chromatid damages were decreased in these cells (Parshad *et al*. 1982). Mannitol also decreases single strand break (SSB) formation and also with binding to SSBs, prevents double strand break formation. This effect probably is due to protection of supercoiled-DNA from relaxation as well as radical scavenging effect of mannitol (Blazek *et al*. 1988).

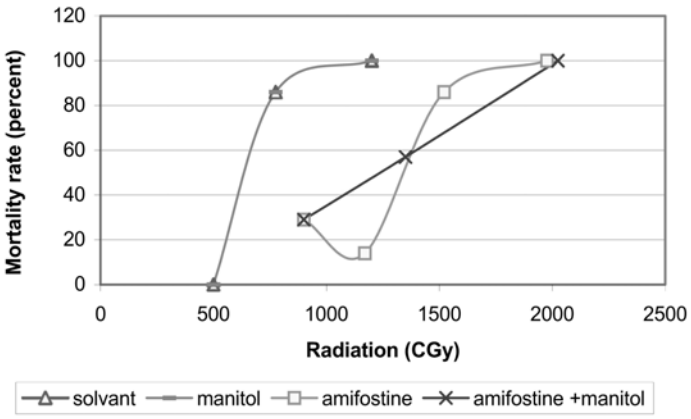

Figure1. Mortality rate in mice exposed to various doses of Gamma radiation in the presence of mannitol, amifostine or combination of both drugs.

Protective effect of mannitol was observed for both low-LET radiations such as x or γ rays, (Peak *et al.*1990, Sagone *et al.* 1983, Parshad *et al.* 1982) and high-LET radiations such as alpha and neutrons (Peak *et al*. 1995, Shao *et al*. 2000).

Mannitol can prevent radiation induced OH° formation in benzoate, deoxyribose and some aminoacids, significantly (Mori *et al.*1993). Some papers emphasize that mannitol is a competitive agent to DNA-ligands as radioprotectors (Martin *et al*. 1992).

Also mannitol causes removal of G2 blockage, which has been formed in HeLa-cells exposed with a tumor – promoter agent. This mannitol effect causes decrease of DNA-damage in cells (Kinzel *et al*. 1985). But, mannitol didn't show free radical scavenging effect in therapeutic dose (Gillbe *et al.* 1996).

In this study, we observed that mannitol didn't decrease the cytotoxic or genotoxic effect of γ - radiation significantly. Because of the intracellular movement and simple diffusion in the cell, mannitol can only neutralize self-inside free radicals (Gillbe *et al*. 1996); so, in low doses of radiation, free radical scavenging effect of this drug is limited. Also, mannitol in high doses is toxic and cause toxicity in kidney leading to acute renal failure (Rabertoy *et al*. 1993).

Mannitol causes prevention of radiation induced G2 blockage and mitotic delay, but as observed the dose of mannitol used in this study did not change the radiation induced proliferation suppression in bone marrow (table 1).

Mannitol washouts from blood faster than amifostine but largest amount of this drug is metabolized in liver (Heine *et al.* 1970). Concurrent application of mannitol with amifostine could not affect on amifostine radioprotection.

In conclusion mannitol with a dose of 400mg/kg has not any radioprotective effect or effects on amifostine radioprotection, and could not decrease cellular or genetic toxicity of radiation.

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