

## Amifostine Protects the White Cell Line Against Genotoxic Damage in Patients Undergoing Pelvic Radiotherapy

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### Abstract

X-rays are widely used in the treatment of cancer. Nevertheless, radiation may induce damage to normal tissues, through free radical release and Deoxyribonucleic acid (DNA) damage. Amifostine is a broad spectrum cytoprotective agent with free radical scavenger and DNA repair activity that reduces toxicity during radiotherapy and chemotherapy. The aim of the present in vivo study was to investigate the cytoprotective efficacy of amifostine against chromosomal damage induced on peripheral blood lymphocytes in cancer patients under radiotherapy for pelvic tumors.

The levels of genotoxicity, cytostaticity and cytotoxicity were quantitatively evaluated using the Sister Chromatid Exchange (SCE) methodology, the Proliferation Rate Index (PRI), the Mitotic Index (MI). PBMCs were collected at 0, 5 and 19 days from patients with pelvic tumors undergoing daily fractionated radiotherapy. Five patients received Radiotherapy (RT) alone (Group A) and 5 received RT supported with amifostine (1000 mg sc.) (Group B) before each RT fraction.

A gradual increase of SCEs was noted in group A ( $p=0.03$ ) and a rather stable score in group B by increasing the days of radiotherapy. At day 19 a significant increased SCE number characterized group A ( $p=0.005$ ). The mitotic index was significantly reduced by increasing the days of radiotherapy in both groups A and B ( $p=0.02$  and  $0.01$  respectively) and there was no difference between groups at the various time points. The proliferation rate index was not affected.

Pelvic radiation induces a significant amount of SCEs in PBMCs and reduces the mitotic index, implying a potential leukemogenic and certainly cytotoxic activity. Amifostine daily administration although did not protect against cytotoxic damage it reduced the amount of SCEs suggestive of a protection against the stochastic effects of radiation.

**Keywords:** Amifostine; Radiotherapy; PBMCs; Sister chromatid exchange

### Introduction

Radiotherapy is an established treatment option for a variety of human carcinomas. The curative efficacy of radiotherapy is, however, limited by the toxicities induced in normal tissues. X-rays mediate DNA single and double strand breaks through generation of free radicals that directly interact with the DNA chains. X-ray-induced DNA damage perturbs the G1, S, and G2 phases of the cell cycle. DNA damage, furthermore, activates checkpoints that delay cell cycle progression to allow the recruitment of repair mechanisms [1].

High doses of X-irradiation can lead to apoptosis in a variety of cell types, whether normal or cancer. DNA damage may also result in irreversible cell cycle arrest or reproductive inhibition [2,3] affiliated by markers of senescence [4,5]. Several pathways including mutations of apoptosis-essential genes or anti-apoptotic protein over expression may, however, allow cell survival and progression through the cell cycle. Cell division in the presence of inappropriately repaired DNA could enhance carcinogenesis [6,7].

The development of normal-tissue-selective cytoprotective agents for administration during radiotherapy will reduce the toxicities of radiotherapy, and would allow dose escalation for improved cure rates. Amifostine (WR-2721) is an inorganic triphosphate pro-drug developed by the Walter Reed Army Institute of Research to protect the soldiers in a case of a nuclear war, but received FDA approval as protector against platinum toxicities and radiation induced xerostomia [8,9]. Amifostine is activated (WR-1065 form) by the enzyme alkaline phosphatase [10]

of the endothelium of normal tissues [11,12] immediately after its administration and acts like a scavenger towards the reactive oxygen species (ROS) emerging from radiotherapy or chemotherapy treatment. After further oxidation (WR-1065 WR-33278) it can bind the DNA and accelerate the repair-mechanism [13].

The aim of this study was to examine the protective efficacy of amifostine against radiation induced chromosome damage in Peripheral Blood Mononuclear Cells (PBMCs). Such an activity would encourage the application of amifostine for protection of cancer patients against the leukemogenic and/or carcinogenic effect of radiation. Amifostine mediated protection was evaluated through the analysis of three cytogenetic indicators. The first index, Sister Chromatid Exchange (SCEs), is a physical phenomenon related to the S phase of cell cycle. SCEs occur on a perfectly homologous chromosome locus, and the term refers to the exchange of genetic material between two

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sister chromatids before the cell transits to metaphase. Breaking and re-bonding of double-stranded DNA chains of each sister chromatid are a prerequisite for their creation. It is a sensitive and rapid method for investigating DNA damage caused by carcinogens even at low concentrations [14,15]. The other two indices are the Proliferating Rate Index (PRI) that measures the cytostaticity and the Mitotic Index (MI) for cytotoxicity.

## Materials and Methods

### Groups and treatment

Ten cancer patients with pelvic tumor, five (5) males with prostate cancer and five (5) females with endometrial cancer, age between 55-65 years old and none of them being a smoker or under other anticancer treatment, were recruited in this study. They were randomly divided in two groups; cancer patients under X-ray radiotherapy treatment (RT control group A, n=5, 2 male-3 female) and cancer patients under X-ray radiotherapy treatment with daily amifostine administration 20 min before radiotherapy (RT amifostine group B, n=5, 3 male-2 female). Amifostine was delivered subcutaneously at a dose of 1000mg according to a previously reported dose escalation algorithm [16,17]. Pelvic radiotherapy was delivered with a slightly hypofractionated and accelerated scheme, giving a total of 14 x 2.7Gy fractions, within 18 days, as previously reported [17].

Blood samples were obtained from the donors at three different time points;  $T_0$  = time point before irradiation,  $T_5$  = five (5) days after the first radiotherapy treatment (13.5Gy of total dose) and at  $T_{19}$  = nineteen (19) days after the first radiotherapy treatment (37.8Gy of total radiotherapy dose). The blood was collected in heparinized vacutainer tubes, 6 ml from each donor, for each time point.

The study has been approved by the local Ethics and Scientific Committees.

### Cell culture

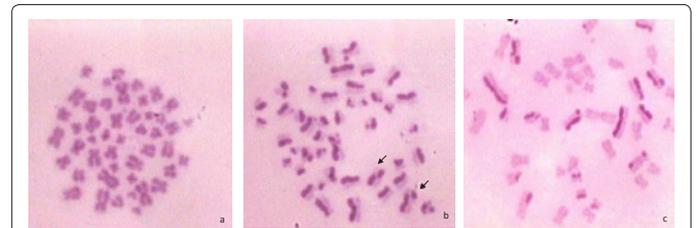
Eleven drops of heparinized whole blood were added to medium culture with phytohemagglutinin in universal containers. For the results of SCE test visualization, 5 µg/ml 5-bromodeoxyuridine was added in the cultures. The cultures were incubated at 37°C for 72h. Two hours before the incubation period, 0.3 µg/ml colchicine (CAS No. 1934-21-0, EEC No. 217-699-5, approx.90% pure) was injected to each culture and at the end of the incubation period, cultures harvested with hypotonic KCl solution and fixed in methanol: acetic acid (3:1, v/v). All chemicals were dissolved in distilled water and the same amount of distilled water was introduced in the control cultures.

### Sister Chromatid Exchanges (SCEs)

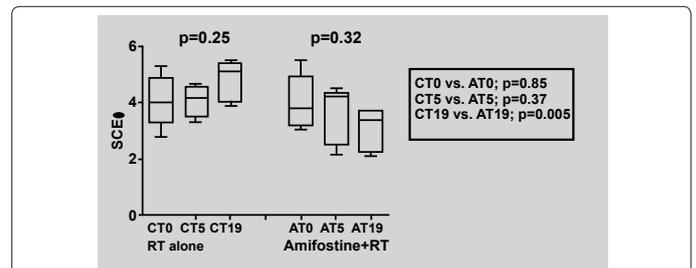
The chromosomes were stained using a modified Fluorescence Plus Giemsa (FPG) technique [14,18-21]. For each culture, 40 well spread second division metaphases were analyzed. Preparations were scored for cells in their first mitosis (both chromatids dark staining), second mitosis (one chromatid of each chromosome dark staining) and third and subsequent divisions (a portion of chromosomes with both chromatids light staining). Slides had been previously coded and scoring was done blindly.

### Proliferation Rate and Mitotic Index (P.R.I & M.I.)

The rate of cell division, the assessment of cell cycle(s) was determined by measuring the proportion of metaphases in first, second



**Figure 1:** Metaphases 1<sup>st</sup> (a-both chromatids dark staining), 2<sup>nd</sup> (b-one chromatid of each chromosome dark staining), 3<sup>rd</sup> (c-a portion of chromosomes with both chromatids light staining). Arrows indicate sister chromatid exchanges.



**Figure 2:** Data graph for sister chromatid exchanges. CT0, CT5, CT19 time points for group A (RT alone); AT0, AT5, AT19 for group B (RT+amifostine).

or third and higher cell division (Figure 1). We used two indices for this estimation. The Proliferation Rate Index (PRI) is calculated according to the formula  $PRI = (1M_1 + 2M_2 + 3M_{3+})/N$  where  $M_1$  is the percentage of cells in the first division,  $M_2$  is the percentage of cells in the second division and  $M_{3+}$  the percentage of cells in the third and higher divisions. Also, N is the total number of metaphases scored for each culture (200 metaphases for each culture). The Mitotic Index (MI) is calculated as the number of metaphases to the number of interphases nuclei (2000 nuclei for each culture)  $MI = N \text{ Metaphases} / N \text{ interphases nuclei} \times 1000$ .

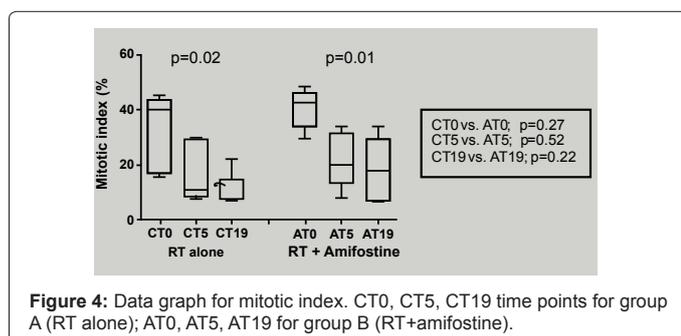
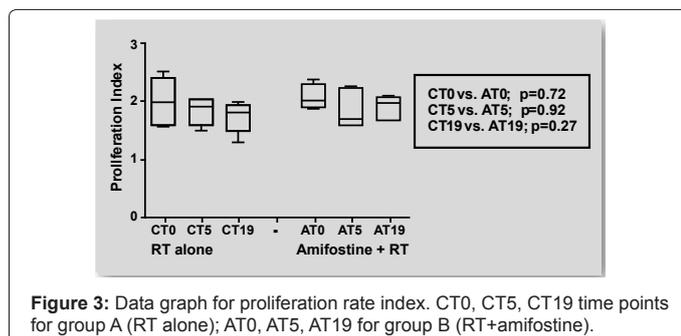
### Statistical analysis

The evaluation of the results calculated mean values, standard deviations and the fixed error of each mean value. As the prices of chromatid exchanges usually do not follow normal distribution, it was necessary to transform them. We used the logarithm transformation and rarely the square root transformation. For comparisons we used the method of analysis of variance one way for independent samples (one way analysis of variance, ANOVA) and the unpaired or paired two-tailed t-test, as appropriate. For statistical analysis, we used the statistical program Graph Pad Prism V 5.0. A p-value <0.05 was used for significance.

## Results

### Sister chromatid exchanges

The statistical analysis with ANOVA test showed that SCEs were not altered statistically neither in group A (RT control group, p=0.25) nor in group B (RT amifostine group, p=0.32). A gradual increase of SCEs was noted in group A and a rather stable score in group B by increasing the days of radiotherapy. Paired two tailed t-test confirmed a significant increased SCE value at day 19 in group A (p=0.03) but not in group B (Figure 2). Comparing the SCEs at day 19 between group A and B (unpaired two-tailed t-test) a significant increased number characterized group A (p=0.005).



### Proliferation Rate Index (P.R.I.)

The proliferation rate index mirrors the cytostaticity; the statistical analysis with ANOVA test demonstrated that no significant alteration was observed neither in the group A ( $p=0.41$ ) nor in the group B ( $p=0.39$ ). By using the unpaired-two-tailed t-test, no statistically significant changes were detected between the two groups at the three time points checked (Figure 3).

### Mitotic Index (M.I.)

Statistical analysis with ANOVA showed a significant reduction of mitosis measurements by increasing the days of radiotherapy in both groups A and B ( $p=0.02$  and  $0.01$  respectively). The unpaired-two-tailed t-test detected a statistically significant decrease of M.I. within group A and B at  $T_{19}$  ( $T_0$  vs.  $T_{19}$ ;  $p=0.003$  and  $T_0$  vs.  $T_{19}$ ;  $p=0.007$ , respectively). There was no difference in the MI values between A and B groups at the various time points (Figure 4).

### Discussion

Surgery, chemotherapy and radiotherapy, whether combined or alone, are the main strategies for anticancer treatment. Radiotherapy of the local and regional disease has been used to treat cancer for more than 100 years. Radiotherapy damages the DNA structure of cells. Photons ionize the intracellular molecules resulting in free radicals that directly interact with DNA strands and produce single and double strand breaks. Normal cells are expected to withstand the effects of radiotherapy, when given at a dose below a certain level, by exploiting mechanisms involved in DNA repair and maintain tissue homeostasis and function. Apart from escaping death, it is vital for normal cells; to fix all radiation induced genetic impairments to avoid the transmission of hereditary abnormalities to the next generation, and also to avoid subsequent leukemogenesis and carcinogenesis. So, during radiotherapy the protection and prevention of any damage of normal cells is a main concern. The first cytoprotective drug that entered clinical trials and was subsequently approved by the FDA for clinical use, was amifostine.

Amifostine is a prodrug that becomes activated when dephosphorylated by alkaline phosphatase of the endothelium of normal tissues. It is converted into an active thiol, an important scavenger for free radicals produced by ionizing radiation or chemotherapeutic drugs that is rapidly distributed within the normal tissues and rapidly absorbed intracellularly [22]. On the other hand, for several reasons [9,23] the active drug does not enter the cancer cell, so protection is normal-tissue-selective.

In this prospective study, we aimed to investigate the *in vivo* cytoprotective effect of amifostine against X-ray induced genotoxic damage, in cancer patients undergoing radiotherapy for pelvic malignancies. We focused on possible alterations on chromosomal structure, in PBMCs of patients. Three major qualitative indices were investigated; the sister chromatid exchange index for genotoxicity, the proliferation rate index for cytostaticity- and the mitotic index for cytotoxicity.

SCEs assessment is a well characterized genotoxicity index. Although, SCEs themselves do not necessarily lead to adverse health outcomes, elevated levels of SCEs apparently indicate increased mutagenic and/or lethal effect of radiation [24]. X-ray induced SCEs were located at the internal regions of chromosomes, though a recent study suggests that chromosome ends are subject to more double-strand breaks during replication and these are more likely to be repaired compared to internal regions of chromosomes [25]. SCEs were gradually increasing during the course of radiotherapy in both the control and the amifostine RT groups, although due to the small number of cases ANOVA test was not significant. Nevertheless, in the group of patients that was not receiving amifostine the SCE induction at 19 days was significantly higher compared to the group receiving amifostine. It was, therefore, concluded that amifostine protected DNA from radiation-induced SCEs. A possible reduction of the SCEs internal rate of development (radiation-independent SCEs) cannot be excluded by the present study. In any case, amifostine decreases genotoxicity caused by X-ray irradiation.

To continue with the second index, namely the proliferation rate indexes (PRI), high levels of PRI imply low cytostaticity. Statistical analysis pointed that any change at the mitosis measurements was not significant or important within both groups involved. Thus, amifostine had no effect on cytostaticity caused by X-rays radiation. In contrast, an *in-vitro* study showed a statistically important higher proliferation rate of endothelial cells following high dose of radiation plus amifostine, compared with radiation alone, and indicating that amifostine might act as an activator of proliferation of the human endothelial cells in a simple *in-vitro* system [26].

Finally the mitotic index (MI) was studied; MI expresses the percentage of cells undergoing mitosis and highlights cytotoxicity. Herein, a statistically meaningful reduction in mitosis measurements within control and amifostine RT groups, especially at  $T_{19}$ . It is suggested that accumulation of DNA damage after multiple radiotherapy fractions may lead to detectable MI reduction. There was no difference in MI measurements between control and amifostine groups at the time points investigated. Recent studies suggest that mitotic delay may be directly induced by amifostine to white blood cells, when the former was administrated combined with specific chemotherapeutic agents [27]. This, however, could not be investigated in the current study as a group of patients receiving amifostine without radiation was not ethical to recruit.

It is concluded that pelvic radiation induces a significant amount

of SCEs in PBMCs and certainly an important reduction of the mitotic index, implying a potential leukemogenic and certainly cytotoxic activity. Amifostine daily administration although did not protect against cytotoxic damage (in any case lymphocytes are among the most radiosensitive cells) it certainly reduced the amount of SCEs suggestive of a protection against the stochastic effects of radiation (mutations and carcinogenicity). Clinical studies addressing this benefit are difficult to run as the carcinogenicity risk following radiotherapy is as low as 2%. Nevertheless, if amifostine could eliminate this “small percentage” a high number of cancer survivors would be saved from secondary tumors, as radiotherapy produces high cure rates in pelvic and other malignancies.

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