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The Effects of Antioxidants on Radiation-Induced Chromosomal Damage in Cancer and Normal Cells Under Radiation Therapy Conditions

Maria Konopacka[1], Jacek Rogoliński[1] and Krzysztof Ślosarek[2]
[1]*Center for Translational Research and Molecular Biology of Cancer,*
[2]*Department of Radiotherapy and Brachytherapy Treatment Planning,*
Maria Sklodowska-Curie Memorial Cancer Center and
Institute of Oncology, Gliwice,
Poland

1. Introduction

Radiotherapy is the major form of treatment for many human cancer. During the course of treatment, the ionising radiation produces many biological effects not only in cancer but also in normal cells. Due to the risk of toxicity to normal cells, the radioprotectors are needed to reduce the normal tissue injury during the irradiation of tumours without influence on effectiveness on cancer treatment. Most of the compounds that showed the radioprotective capacity in laboratory studies failed because of their toxicity to the normal cells. The good radioprotector should be non-toxic and should selectively eliminate the cancer cells. A number of natural dietary ingredients show capacity to protect cells from damage induced by ionising radiation (Arora et al., 2008).

It is well known that antioxidant vitamins such as ascorbic acid and vitamin E protect cellular DNA and membranes from radiation-induced damage (Noroozi et al., 1998; Konopacka & Rzeszowska-Wolny, 2001; Kumar et al., 2002; Jagetia, 2007). Recently, several flavonoids, polyphenols and phenolic acids have become more popular as diet compounds due to their beneficial impact on human health. One of them is ferulic acid. It renders preferential radioprotection to normal tissue, but not to tumour cells under both ex vivo and in vivo conditions (Maurya et al., 2005; Maurya & Nair, 2006). Ferulic acid is present in many plant products such as giant fennel, green tea, coffee beans and grains. It is monophenolic phenylpropanoid that acts as an antioxidant against peroxyl radicals-induced oxidation in neuronal culture and in synaptosomal membranes (Kanski et al., 2002). It was found that ferulic acid reduced the number of radiation-induced DNA strand breaks and enhanced the DNA repair processes in peripheral blood lymphocytes but not influenced the level of radiation-induced damage in fibroblastoma tumour cells in mice (Maurya et al., 2005).

It has been also showed that the α-tocopherole can preferentially reduce the level of radiation-induced chromosomal damage in normal human cells, but in cancer cells it

actually increases the level of damage (Jha et al., 1999; Kumar et al., 2002). Although there is much evidence about the modulating effects of antioxidants in cells directly irradiated the effects of low dose of scattered radiation are poorly studied.

Our previous study showed that the cells placed outside the irradiation field were exposed to very low doses of scattered radiation that induced the micronuclei (Konopacka et al., 2009) and decreased the cell viability (Rogolinski et al., 2009). The extent of micronuclei formation and apoptosis as well as the decrease of viability of cells exposed to scattered radiation was higher that could be predicted by dosimetric methodology based on the linear non-treshold model (LNT). In this situation, the radioprotection of normal tissues placed outside the irradiation field during radiotherapy of cancer seems to be very crucial. In present study, we compared the influence of vitamin C, vitamin E and ferulic acid on micronuclei formation in human cancer cells directly irradiated and in normal human cells placed outside the irradiation field during exposure. This study was performed to answer the question whether the antioxidants selected by us could preferentially protect normal cells but not cancer cells during exposure to radiation in conditions mimicking the radiotherapy. We tested the modulating effects of vitamin C, vitamin E and ferulic acid on micronuclei formation in directly irradiated cancer A549 cells as well as in normal BEAS-2B cells exposed outside of the radiation field.

2. Experimental procedures

2.1 Cell culture

Human lung carcinoma cells (A549 line) and normal human bronchial epithelial cells (BEAS-2B) were grown on DMEM/F12 medium supplemented with 10% fetal bovine serum (Immuniq) in a humidified atmosphere of 5% CO_2 at 37^{o}C. Before irradiation the cells were trypsinized and 20 µl of cell suspension, containing approximately 10^3 cells, were transferred to an Eppendorf`s tubes and then the tubes were filled with medium up 0.5 ml so the cells were irradiated without presence of air.

2.2 Preparation of antioxidants

Vitamin C (Serva, Germany) was dissolved in culture medium and filter sterilized. Vitamin E (α- tocopherole, Sigma) and ferulic acid (Linegal) were dissolved in ethanol. Antioxidants were added to cultures 1 h before irradiation at final concentration from 1 to 100 µg/ml. Experiments included control cultures treated with ethanol alone at the same volume as volume of vitamin E or ferulic acid added to cultures.

2.3 Irradiation of cells

The cancer cells in Eppendorf`s tubes were placed in a special stand on 3 cm of depth in a water phantom and expose to 5 Gy. The normal cells in Eppendorf`s tubes were placed in a water phantom at distance of 4 cm outside the radiation field. These cells were exposed to scattered radiation at dose of 0.2 Gy. The tubes were placed horizontally in the water phantom in such a way that the A549 cancer cells were within the radiation beam field, whereas the normal BEAS-2B cells were placed 4 cm outside the beam field, as is presented in Fig.1.

Experiments were performed for electron (22 MeV) radiation generated in a linear accelerator Clinac series Varian Medical system, for 300 Mu/min accelerator mode and dose

of 5 Gy in build-up (3 cm) depth in a water environment. After irradiation the cells were transferred into plastic dishes (50 mm diameter) and supplemented with up 5 ml of the culture medium.

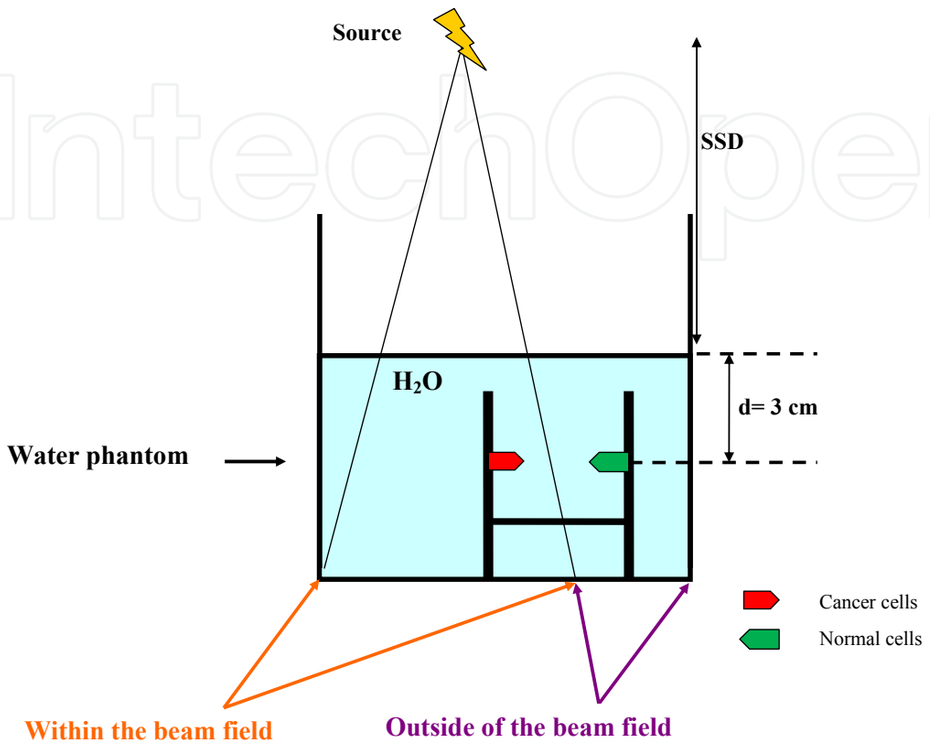


Fig. 1. The scheme of the irradiation set-up

2.4 Cytokinesis-block micronucleus test

The micronucleus test was performed according to the standard procedure (Fenech & Morley, 1985). After irradiation and transferring of cells to culture dishes, the cytochalasin B was added to medium to a final concentration 2 μg/ml and cells were incubated for 48h prior fixation. The cells were fixed *in situ* with a cold solution of 1 % glutaraldehyde (Sigma) in phosphate buffer (pH=7.5) and stained by Feulgen reaction. At least 500 binucleate cells were examined for the presence of micronuclei (MN-BN cells) under microscope.

2.5 Statistical analysis

Experiments were repeated three times. Means ± SD were calculated from experimental data and the Student`s t-test was used to determine the statistical significance of differences in the number of micronuclei between cells cultured and irradiated in the presence or absence of antioxidants.

3. Results

The effect of ferulic acid, vitamin C and vitamin E on the micronuclei formation in the normal human BEAS-2B cells is presented in Tab.1.

Concentration (μg/ml)	Frequency of MN-BN cells (%)		
	Ferulic acid	Vitamin C	Vitamin E
0	3.83 ± 0.44		
1	3.50 ± 0.32	3.44 ± 0.28	3.75 ± 0.43
0	3.33 ± 0.40	3.50 ± 0.42	3.80 ± 0.53
50	4.00 ± 0.62	3.85 ± 0.49	3.66 ± 0.44
100	4.98* ± 0.66	4.00 ± 0.55	4.10 ± 0.58
Ethanol	3.90 ± 0.42		

*- significantly different from control at $p < 0.01$.

Table 1. Influence of ferulic acid, vitamin C and vitamin E on the micronucleus formation in BEAS-2B cells. Means ±SD of three experiments are shown.

The background level of micronuclei was 3.83 ± 0.44 and none of the antioxidants at their concentration below 50 μg/ml caused any changes above this value. When the concentration of antioxidants was increased to 100 μg/ml the frequency of micronucleated cells was higher in comparison with background value but this difference was significant only for ferulic acid. Ethanol used as a solvent of ferulic acid and vitamin E did not change the spontaneous level of micronuclei in BEAS-2B cells.

Concentration (μg/ml)	Frequency of MN-BN cells (%)		
	Ferulic acid	Vitamin C	Vitamin E
0	2.10 ± 0.32		
1	2.18 ± 0.35	2.21 ± 0.33	2.25 ± 0.33
10	6.62* ± 0.55	2.26 ± 0.36	2.27 ± 0.20
50	9.16** ± 0.74	2.16 ± 0.29	2.20 ± 0.29
100	10.20** ± 0.98	2.23 ± 0.25	2.34 ± 0.42
Ethanol	2.16 ± 0.47		

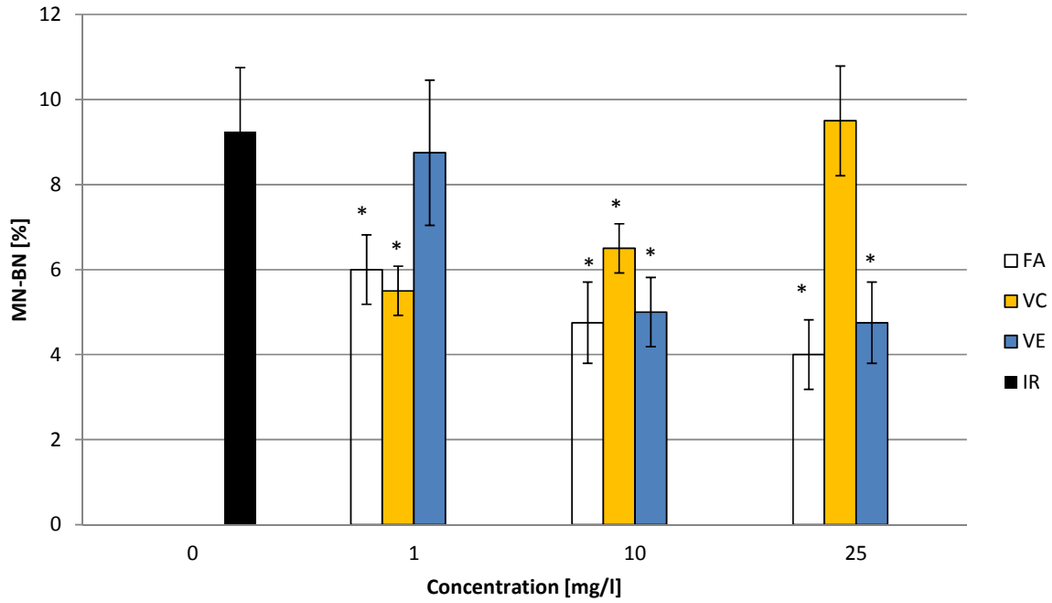
Significantly different from control at: * $p < 0.01$, **$p < 0.001$.

Table 2. Influence of ferulic acid, vitamin C and vitamin E on the micronucleus formation in A 549 cells. Means ±SD of three experiments are shown.

Tab.2 presents the effect of antioxidants on the micronuclei formation in tumour A549 cells. Addition of vitamin C or vitamin E at concentrations ranging from 1 up to 100 μg/ml did not cause any measurable changes in the spontaneous level of micronuclei (2.10 ± 0.32). Ferulic acid at the concentrations 10 μg/ml and higher increased significantly the number of micronucleated cells in comparison with the control cells incubated without antioxidants. Ethanol did not cause any changes above the background in A549 cells.

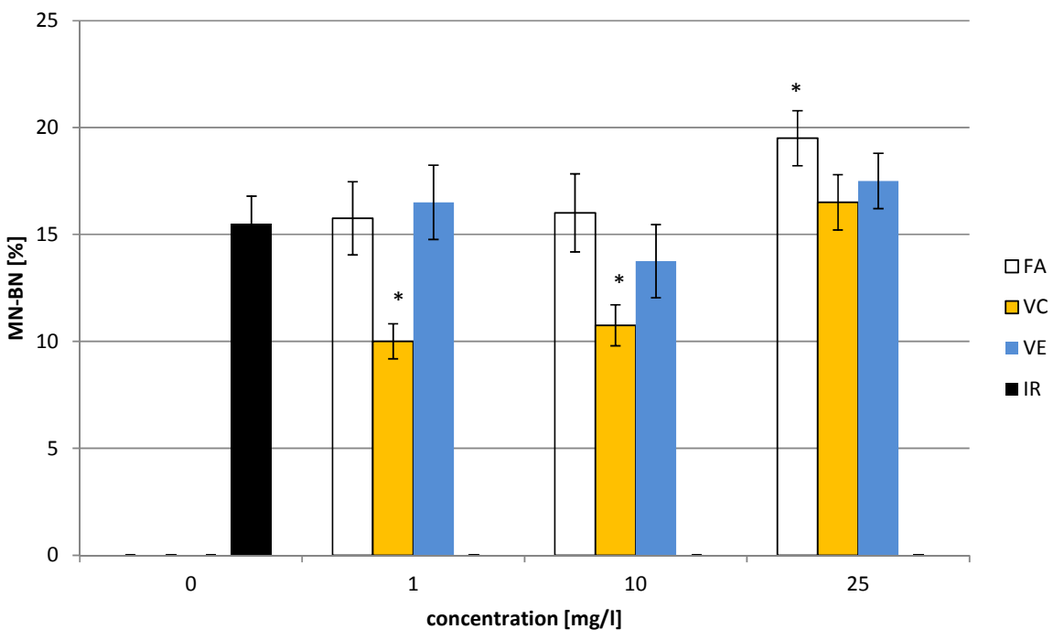
In the next experiments we tested the effect of antioxidants on the level of radiation-induced micronuclei in cells irradiated in a water phantom (see Material and Methods). Antioxidants were tested at concentrations 1, 10 and 25 μg/ml. The results of this experiment in normal BEAS-2B cells exposed to radiation outside the field are showed in Fig.2.

Vitamin C at the concentrations of 1 and 10 μg/ml protected cells from radiation-induced DNA damage but at concentration of 25 μg/ml it was not effective in reducing this damage. In contrast to ascorbic acid, vitamin E was effective as a radioprotector at concentration above 10 μg/ml. Ferulic acid showed the best protective effect against radiation-induced micronuclei in normal cells. It inhibited significantly the micronuclei formation in dose-dependent manner.



Values are means ±SD of three experiments.
* $p < 0.01$ refers to differences between irradiated only and preincubated with antioxidants cells (Student's t – test).

Fig. 2. Effect of ferulic acid, vitamin C and vitamin E on the micronuclei formation in BEAS-2B cells exposed to radiation outside the field.



Values are means ±SD of three experiments.
* $p < 0.01$ refers to differences between irradiated only and preincubated with antioxidants cells (Student's t – test).

Fig. 3. Effect of ferulic acid, vitamin C and vitamin E on the micronuclei formation in A-549 cells irradiated in a beam axis.

Fig.3 shows the radioprotective capacity of antioxidants in cancer cells irradiated in beam of axle. Vitamin C at low concentration (below 10 μg/ml) diminished the level of radiation-induced micronuclei. At the concentration of 25 μg/ml we observed no effect of this vitamin on formation of micronuclei in irradiated cells in comparison with the cells irradiated without vitamin treatment. Vitamin E at all concentrations did not influence the level of radiation-induced chromosomal damage. In cells irradiated in the presence of ferulic acid at concentrations 1 or 10 μg/ml we did not observe any protective effect in reducing the level of radiation-induced micronuclei but at the highest concentration (25 μg/ml) ferulic acid significantly increased the number of radiation induced micronuclei.

4. Discussion

Radioprotectors should selectively protect the normal tissues during radiotherapy of cancer without inhibition of damaging of cancer cells. In present work we tested the radioprotective activity of known antioxidants, namely, ferulic acid, vitamin C and vitamin E, in normal cells versus cancer cells. To answer the question whether these antioxidants can preferentially protect the normal cells during radiotherapy, we performed experiments under conditions like those that would be utilized in radiation therapy treatment procedures: tumour cells were irradiated directly (in a beam axle), whereas the normal cells were placed outside the radiation field during exposure. Our results indicate that the radioprotective effect of vitamin C is concentration-dependent and similar in normal and cancer cells; vitamin C at low concentration diminished the radiation-induced micronuclei whereas at high concentration it enhanced the level of damage, that was stronger in cancer than in normal cells. It is known that high concentration of ascorbic acid can potentate the production of hydroxyl radicals from hydrogen peroxide via Fenton reaction, which enhances the level of radiation-induced DNA damage (Halliwell & Gutteridge, 1985). This action of vitamin C varied among cell types due to the differences in the intracellular concentration of the ascorbic acid ranging from 10 μM in serum blood cells to 700 μM in bone marrow cells (Umegaki et al., 1995). It has been showed that vitamin C supplemented with vitamin K3 was effective in killing of cancer cells via activation of DNase, which degrades tumour cell DNA and induces cell death (Jamison et al., 2004).

Our results indicate that ferulic acid and vitamin E are potentially very good radioprotectors of normal cells during radiotherapy because they reduced the number of radiation-induced micronuclei in normal cells and simultaneously they did not influence the damaging effect of radiation in cancer cells and at high concentration they enhanced the damaging effect of radiation in cancer cells. Moreover ferulic acid showed selectively clastogenicity expressed as a micronuclei formation in tumour cells. The number of micronucleated cancer cells increased over the background value in concentration-dependent manner.

Our observations are in agreement with published data that indicated that ferulic acid preferentially protected normal mouse bone marrow and blood cells but not cancer fibroblastoma cells in mice exposed to 4 Gy of γ-radiation (Maurya & Nair, 2006). It was also shown that it protected human lymphocytes against radiation-induced chromosomal damage (Prasad et al., 2006). Moreover, it was found that vitamin E selectively protected normal human fibroblasts but not human cervical cancer and ovarian carcinoma cells against radiation-induced chromosomal damage (Kumar et al., 2002) and cell cycle inhibition (Jha et al., 1999). It has been suggested that the preferential protection of normal cells against radiation can be connected with alteration of genes encoding the elements of

the cell signalling pathways such as transcriptional factor E2F (Turley et al., 1997) and repair processes (Maurya & Nadir, 2006).

5. Conclusions

The results presented in this paper indicate that antioxidants such as ferulic acid and vitamin E protect to normal cells exposed to low dose of scattered radiation present outside the radiation field. Recently, there is increasing attention for the low dose radiation exposure including non-target phenomena such as bystander effect and low dose hypersensitivity and genetic instability (Morgan & Sowa, 2007) that can be responsible for the induction of secondary cancer. The protection of normal cells against these distant effects appears to be important element in radiotherapy and out to be taken into consideration in clinical practice. Ferulic acid and vitamin E seem to be the promising protectors of normal cells during radiotherapy.

6. Acknowledgment

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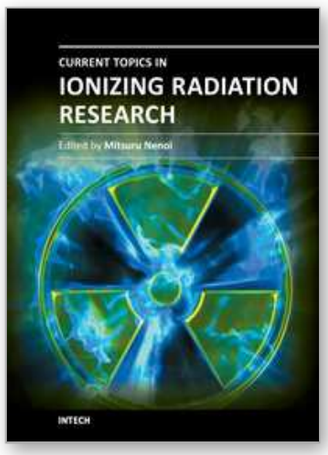
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Since the discovery of X rays by Roentgen in 1895, the ionizing radiation has been extensively utilized in a variety of medical and industrial applications. However people have shortly recognized its harmful aspects through inadvertent uses. Subsequently people experienced nuclear power plant accidents in Chernobyl and Fukushima, which taught us that the risk of ionizing radiation is closely and seriously involved in the modern society. In this circumstance, it becomes increasingly important that more scientists, engineers and students get familiar with ionizing radiation research regardless of the research field they are working. Based on this idea, the book "Current Topics in Ionizing Radiation Research" was designed to overview the recent achievements in ionizing radiation research including biological effects, medical uses and principles of radiation measurement.

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中国上海市延安西路65号上海国际贵都大饭店办公楼405单元
Phone: +86-21-62489820
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