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The radiorecovery potential of nutraceuticals in cellular defense after ionizing radiation *in vitro*

A. LESKOVAC[1], S. PETROVIC[1], A. VALENTA-SOBOT[1], I. JOKSIC[2], M. BALTIC[3], L. ALEKSIC[3], G. JOKSIC[1]

[1]Vinca Institute of Nuclear Sciences, University of Belgrade, Belgrade (Serbia)
[2]Clinic of Gynecology and Obstetrics "Narodni Front", Belgrade (Serbia)
[3]Oncology Institute of Vojvodina, Sremska Kamenica (Serbia)

Abstract. – *Background:* **The aim of this study was to evaluate the effects of two nutraceuticals Amazon Megamin and Immunarc forte in radiorecovery of human lymphocytes after exposure to ionizing radiation *in vitro*.**

Material and Methods: **The incidences of micronuclei, cell proliferation, apoptosis and lipid peroxidation products were examined in cultured human peripheral blood lymphocytes before and after ionizing radiation in a present of nutraceuticals *in vitro*.**

Results: **Results showed that Amazon Megamin and Immunarc forte possess antioxidant properties; they act by eliminating the toxic metabolites, scavenging the free radicals and decreasing lipid peroxidation.**

Conclusion: **The obtained results indicated that the studied nutraceuticals can help in prevention of the development of injurious caused by ionizing irradiation and, therefore, they encourage studies on their radioprotective properties.**

Key Words:

Amazon Megamin, Immunarc forte, Radiorecovery.

Introduction

Over the past few decades, researches in the development of radioprotectors worldwide have focused on screening a plethora of chemical and biological compounds. The large numbers of drugs of both synthetic and natural origin have been tested in *in vitro* and *in vivo* models, and in the human clinical trials to mitigate injuries caused by ionizing radiation[1-5]. However, because of the inherent toxicity at useful concentrations, none of them could find clinical acceptance[6,7]. The development of effective radioprotectors and radiorecovery drugs is of great importance in view of their potential application during the radiation exposure.

The interaction of ionizing radiation with living cells induces a variety of reaction products and a complex chain reaction in which many macromolecules and their degradation products participate[8-13]. These effects are conventionally attributed to an irreversible change resulting from the deposition of energy in the DNA of an irradiated cell being fixed either during the processing and enzymatic repair of DNA damage or during DNA replication[6]. Interaction of ionizing radiation with the biological system results in the generation of many highly reactive short-lived reactive oxygen species (ROS), mainly due to the hydrolysis of water[14]. These ROS then attack cellular macromolecules like DNA, RNA, proteins and membranes, causing their dysfunction and damage[15,16]. Because ROS produced during exposure to ionizing radiation perturb the integrity and survival of cells, the possible mechanisms of defense against ionizing radiation could be modifying signal transduction pathways, redox states, and the disposition of cells to apoptosis[17]. Nature has provided an abundance of natural antioxidant nutrients. The great potential of polyphenols from plant, vegetables and fruits, algal, as well as some less known as zeolites, in action against cells injury is highlighted. Many researchers have recently tested the activity of such products and the possible mechanisms of their anticancer action[18-24]. Most of such products act as potential antioxidants.

In this study, we examined the effects of two nutraceuticals Amazon Megamin and Immunarc forte. Radiation-induced micronuclei formation, cell proliferation and induction of apoptosis in

Corresponding Author: Gordana Joksic, MD; e-mail: gjoksic@vinca.rs

cultured human peripheral blood lymphocytes were studied. As a marker of oxidative stress in irradiated cells we measured malondialdehyde levels.

Material and Methods

Nutraceuticals

Amazon Megamin (Biofarm, Belgrade) consists of a naturally zeolite clinoptilolite (aluminum silicate) in micronizated form tagged with vitamins and minerals all preserved in honey. Amazon Megamin was dissolved in sterile double-distilled water, kept for 30 minutes at room temperature and filtered using 0.45 μm membrane filters (Millipore Co., Ltd.; Billerica, MA, USA).

Immunarc forte (Biofarm, Belgrade) which consist of *Phaeophycea digitata* Khorbi (2.00 mg), *Echinacea purpurea* (90.00 mg), germanium 132 (1.50 mg), nicotinic acid (8.00 mg), beta-carotene (0.16 mg), pyridoxine (1.00 mg), tocopherol (60.00 mg), and acidum ascorbicum (12.00 mg) packed in capsules was dissolved in sterile double-distilled water, kept for 30 minutes at room temperature and filtered using 0.45 μm membrane filters (Millipore Co., Ltd.; Billerica, MA, USA).

Irradiation Procedure

Peripheral blood used in experiment was obtained from twenty healthy, non-smoking young male donors. Blood samples were attained during routine yearly health checkup at the Medical Unit in accordance with current Health and Ethical regulations in Serbia[25]. Aliquot of heparinized whole blood was put into sterile plastic test-tube, placed in a Plexiglas container 15x15 cm and irradiated using ^{60}Co γ-ray source. The radiation dose employed was 2 Gy, the dose-rate 0.45 Gy/min, the dimensions of radiation field were 20 × 20 cm, and the distance from the source was 74 cm. Blood samples were irradiated at room temperature and were set up in cultures one hour after irradiation.

Cell Cultures

Aliquots of irradiated whole blood (0.5 mL) were placed in cultures containing PB-max karyotyping medium (Invitrogen-Gibco, Paisley, UK). Three parallel sets of blood cultures were set up in the presence of increasing doses of Megamin (final concentration of 2 mg/mL, 4 mg/mL, 6 mg/mL and 10 mg/mL, respectively) and Immunarc (final concentration of 0.5 mg/mL, 1 mg/mL and 1.5 mg/mL, respectively) and, mixture of nutraceuticals (Megamin and Immunarc) in final concentrations of 2 mg/mL+0.5 mg/mL, 2 mg/mL+1 mg/mL and 2 mg/mL+1.5 mg/mL, respectively.

For the evaluation of the synergistic effect of Megamin and Immunarc on the cell cycle, nutraceuticals were added in cell cultures at different phases of the cell cycle. G1 phase is considered as the beginning of the cell culture. For studying effects of nutraceuticals on S phase cells, treatment was performed 24 h after culture initiation. Cells in G2 phase of the cell cycle were treated 69 h after culture initiation.

The most thoroughly investigated radioprotective drug, Amifostine (purity 98%; WR-2721, Military Technical Institute, Belgrade, Serbia) was used as positive control. Dose of 100 μL of Amifostine was used for treatment of irradiated cell cultures (final concentration 0.5 μg/mL per culture). Cells grown in the absence of nutraceuticals served as the negative control.

Micronucleus Assay

For micronuclei (MN) preparation, the cytokinesis block method was followed[26]. Cytochalasin B at a final concentration of 4 μg/mL was added to each culture after 44 hours of incubation to inhibit cytokinesis. The lymphocyte cultures were incubated for further 28 hours. Cells were collected by centrifugation and treated with hypotonic solution at 37°C. Hypotonic solution consisted of 0.56% KCl+0.90% NaCl (mixed in equal volumes). Cell suspension was fixed in methanol/acetic acid (3:1), washed three times with fixative and dropped onto clean slide. Slides were air dried and stained in alkaline Giemsa (2%).

Slide Scoring

All slides were analyzed with an AxioImager A1 microscope (Carl Zeiss, Jena, Germany) using magnification 400 ×, or 1000 × when necessary. A minimum of 1000 binucleated cells were scored to evaluate the percentage of cells with one, two three, four or more than four micronuclei.

A cytokinesis-block proliferation index (CBPI) was calculated with the equation: CBPI = [MI+2MII+3(MIII+MIV)]/N, where MI-MIV represents the number of cells with one to four nuclei, respectively, and N is the number of cells scored[27].

Thiobarbituric Acid (TBA) Assay

After 72 hours of incubation, parallel cultures were separated on Lymphoprep, lymphocytes were collected by centrifugation, washed in physiological saline, and frozen at –20°C. MDA was determined by measuring the chromogen obtained from the reaction of MDA with 2-thiobarbituric acid[28,29]. Defrosted lymphocytes suspension (0.1 mL) was added to 0.4 mL 50 mM Tris-HCl buffer containing 180 mM KCl and 10 mM EDTA, final pH 7.4. To 0.5 mL of prepared sample were added 0.5 mL of 2-thiobarbituric acid (Merck, 1% wt/vol) in 0.05 M NaOH and 0.5 mL of HCl (25% wt/vol in water). The mixture was placed in glass tubes, and heated in boiling water for 10 min. After cooling, the chromogen was extracted in 3 mL of *n*-butanol and the organic phase was separated by centrifugation at 5000 rpm for 10 min. The absorbance of the organic phase was measured spectrophotometrically (Lambda 35 UV/Vis spectrophotometer, PerkinElmer Life and Analytical Sciences, Inc., Waltham, MA, USA) at 532 nm wavelength. The values are expressed as nmol of thiobarbituric acid-reactive substances (TBARS) (MDA equivalent)/mg of protein, using a standard curve of 1,1,3,3-tetramethoxypropane. Protein concentration was determined according to Lowry et al[30].

Apoptosis Assay

For the apoptosis (AP) assay, 0.5 mL irradiated whole blood from each blood donor were added into 4.5 mL RPMI-1640 medium supplemented with 15% of calf serum without phytohemagglutinin (PHA) and treated with nutraceuticals at investigated concentrations. Inherent irradiated control was made. After incubation in CO_2 incubator for 24 hours, cells were gently washed with physiological saline (0.9% NaCl) at 37°C, and fixed in methanol:acetic acid (3:1)[31]. Afterwards, the pellet was fixed in 96% ethanol and frozen at –20°C. Defrosted samples were incubated at room temperature for 10-15 min in incubation buffer (5 mM $CaCl_2$, 140 mM NaCl, 10 mM 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid (HEPES)/NaOH pH 7.4). Propidium iodide (PI) was added 5 min prior to flow cytometric analysis (final concentration 1 μg/mL). Samples were analysed by flow cytometry (Becton Dickinson, Heidelberg, Germany). Apoptotic population was calculated using the CellQuest software (Becton Dickinson).

Statistics

Statistical analysis was carried out using the statistical software package Statistica 8, and OriginPro 8 for Windows Vista. Results were expressed as percentages of control. $p < 0.05$ was considered to be significant.

Results

The effects of Megamin on irradiated lymphocytes are presented in Figure 1. These include the incidence of radiation-induced micronuclei (MN), proliferation potential of cells, level of malondialdehyde (MDA) and, percentage of apoptotic cells (AP). Megamin at lowest concentration (2 mg/mL) markedly decreased the frequency of MN (38.42%) as well as level of MDA (25.06%) and increased percentage of apoptotic cells (41.25%) compared to the unexposed lymphocytes. Treatment of irradiated human lymphocytes with Megamin (concentration 4 mg/mL) decreased the incidence of MN by 23.05%, level of MDA by 14.47%, whereas the percentage of apoptotic cells was enhanced by 30.76%. Further increases in concentration of Megamin (from 6 mg/mL to 10 mg/mL) slightly increased the frequency of micronuclei and level of MDA without significant influence on percentage of apoptotic cells. Proliferation potential of cells remained almost unchanged with increasing the dose.

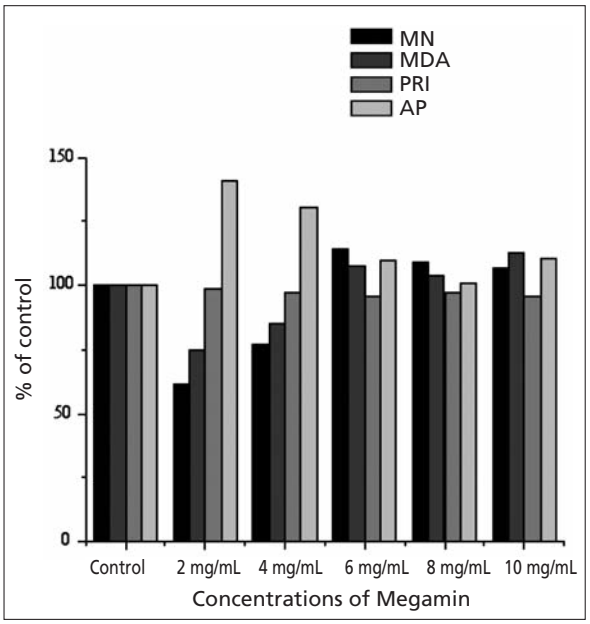

Figure 1. Percentage of micronuclei, level of malondialdehyde and percentage of apoptotic cells in irradiated human lymphocytes treated with different concentrations of Megamin.

Treatment of irradiated human lymphocytes with Immunarc (Figure 2) resulted in significant decrease of the frequency of MN as well as level of MDA in a dose-dependent manner. Immunarc at concentration of 0.5 mg/mL reduced the frequency of MN by 17.23%, increased the percentage of cells undergoing apoptosis by 28,32% and affected lipoperoxidation processess on cell membranes reducing the level of MDA by 57.88% compared to the control. The similar results were obtained in treatment of cells (PRI) with concentration 1 mg/mL, whereas at concentration of 1.5 mg/mL were observed toxic effects seen as increasing the frequency of MN by 35.55% and level of MDA by 36.95%. The proliferation potential of cells (PRI) was reduced at all examined concentrations, statistically insignificant.

Treatments of irradiated human lymphocytes with various concentrations of Megamin and Immunarc are presented in Figure 3. All examined combinations reduced incidence of MN as well as levels of MDA and, increased percentage of apoptotic cells. The best effect was obtained by synergistic action of 2 mg/mL of Megamin and 1 mg/mL of Immunarc. Frequency of radiation-induced MN was reduced by 50.91%, level of MDA was reduced by 36.61%, whereas the percentage of apoptotic cells was enhanced by 41.38%. Proliferation potential of cells was slightly reduced in respect to the control, with no statistical significance.

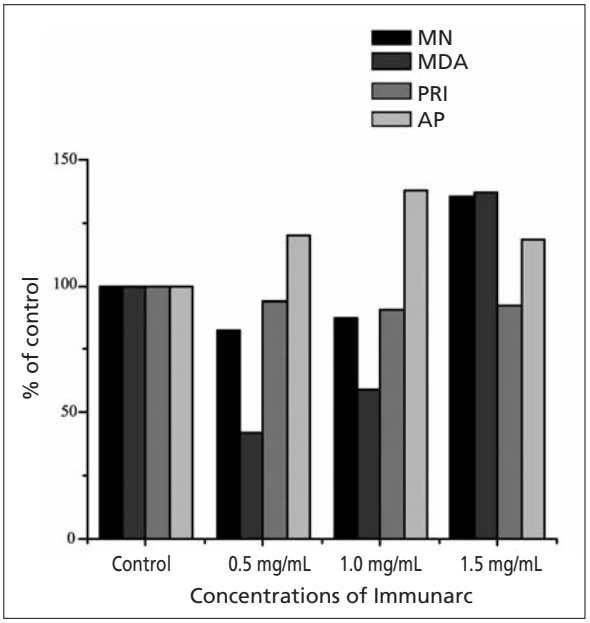

Figure 2. Percentage of micronuclei (MN), proliferation potential of cells, level of malondialdehyde and, percentage of apoptotic cells in irradiated human lymphocytes treated with different concentrations of Immunarc.

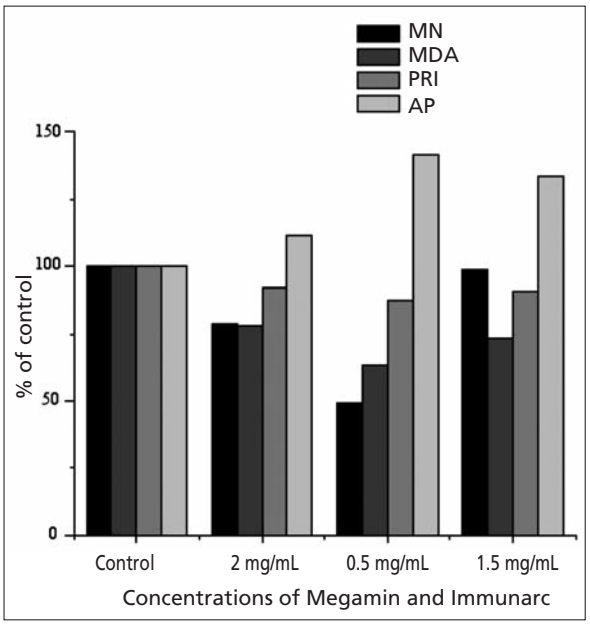

Figure 3. Percentage of micronuclei (MN), level of malondialdehyde, proliferative index (PRI) and percentage of apoptotic cells in irradiated human lymphocytes treated with different concentrations of Megamin and Immunarc.

Table I summarizes effects of both nutraceuticals Megamin and Immunarc on irradiated cells treated with nutraceuticals' mixture at different phases of the cell cycle. Based on these results the mixture of nutraceuticals had protective effects in S phase of the cell cycle. Incidence of MN and level of MDA decreased in a dose dependent manner, statistically significant compared to the control ($p < 0.05$). On the contrary, percentage of apoptotic cells significantly increased by 36-69% compared to the control ($p < 0.01$). During the G1 phase of the cell cycle the incidence of MN and level of MDA showed slightly reduction in respect to the control, with no statistical significance. Percentage of apoptotic cells were enhanced by approximately 25% ($p < 0.05$). In G2 phase, there were no significant changes in the examined parameters relative to control. Significant alterations in the proliferation potential of cells during treatment at different cell cycle phases were not observed.

Amifostine (concentration 0.5 μg/mL) which was used as positive control, reduced the fre-

Table I. Incidence of micronuclei (MN), level of malondialdehyde (MDA), proliferative index (PRI) and percent of apoptotic cells (AP) in the G1, S and G2 phases of the cell cycle in irradiated human lymphocytes after treatments with different concentrations of Megamin and Immunarc.

Treatment	Concentrations of Megamin and Immunarc (mg/mL)	MN	MDA	PRI	AP
Control	2 + 0.5	211.53	9.16	1.42	7.05
		184.23	7.80	1.44	8.23
Phase G1	2 + 1.0	192.39	8.77	1.42	7.96
	2 + 1.5	195.13	8.72	1.40	8.90
	2 + 0.5	135.19	4.92	1.44	11.95
Phase S	2 + 1.0	141.25	5.83	1.42	9.85
	2+ 1.5	144.63	5.06	1.43	9.61
	2 + 0.5	198.06	10.01	1.41	7.01
Phase G2	2 + 1.0	208.57	9.69	1.40	6.91
	2 + 1.5	192.11	8.67	1.40	6.84
Amifostine		172.02	8.87	1.44	7.64

quency of MN for 18.68%, level of MDA for 3.17% and enhanced apoptosis for 8.37%. Amifostine did not significantly influence the cell proliferation.

Discussion

The present study of biological effects of Megamin, demonstrated its remarkable radioprotective properties. The active compounds of Megamin reduce the level of radiation-induced DNA damages very efficiently with no adverse effects. Megamin also enhanced apoptosis without disturbing proliferation potential of cells. Furthermore, it displayed strong regenerative properties: reduced the level of free radicals capable of inducing oxidative damages of the DNA and mildly suppressed lipid peroxidation of cell membranes. The powerful potential of Megamin could be attributed to its constituent naturally zeolite clinoptilolite (aluminum silicate). Clinoptilolites in the form of nano-particles can input nutriment into cells and take out noxious products of metabolism from cells. In cell, they release free water from crystal lattice, enhance potential of cell membrane and change its semi permeability[32,33]. Some previous studies indicated that zeolites possess anticancer activity modifying the activity of one or more protein kinases involved in cell cycle control. Kinases such as protein kinase A, B, C, JNK-1, CDK-2, and CDK-4 are either activated or deactivated by these antioxidants. This can happen directly or indirectly through activation of some transcription factors NF-IL6 or tumor suppressor genes, p21 and p27[34].

This study has shown that the Immunarc at low concentrations reduces the radiation-induced micronuclei and the level of MDA. The significant reduction of level of MDA showed the protective effect of Immunarc against lipoperoxidative damage. Results suggest that Immunarc acts on cell membrane processess via eliminating the toxic metabolites, scavenging the free radicals and decreasing lipid peroxidation. Immunarc is mixture of alga *Laminaria digitata* and *Echinacea purpurea* plant extracts and has prediction to be scavenger. According to literature data, algal extracts have demonstrated antioxidative and antitumoral activities on mammals[35,36], and for edible brown algae *Laminaria digitata*, antioxidant and antimutagenic properties have been identified.[37-39] *Echinacea purpurea* has powerful radioprotective capacities, possesses immunomodulatory properties that alleviate apoptosis and enhance monocyte-macrophage activity[40]. Immunarc possesses optimal concentration of vitamins B3, B6, A, E and C, all of which are essential coenzymes for DNA repair enzymes, keeping them in the most efficient state. Its antimutagenic compounds efficiently remove free radicals generated inside the cells and preserve the integrity and functionality of cell membrane enabling proper signal transduction inside and among the cells in

tissue. Immunarc displays strong regenerative properties. Biologically active compounds of Immunarc remove free radicals from cells, significantly reduce lipid peroxidation of cell membranes, and stimulate activity of cellular immunity.

In this study, we have also examined synergistic effects of Megamin and Immunarc to screen influence on cell cycle during three different treatments as well as contribution of each nutraceuticals on cell recovery. Cell cycle checkpoints have been considered important molecular targets for modulation of radiation response[41]. Ionizing radiation affects cells at any stage of the cell cycle, but cells are most vulnerable in G1 and S phases. In the different phases in which simultaneous treatments were performed, the best protective effects were evidenced in S phase of the cell cycle. The damaged and nonfunctional cells were eliminated without inflammation and necrosis due to activity of both examined nutraceuticals. Significantly reduced lipid peroxidation by drug combinations seems to be very important in reducing the yield of micronuclei. During G1 phase of the cell cycle, they displayed mild protective effects on irradiated lymphocytes. In G2 phase no significant changes in examined parameters compared to both positive and negative control were observed.

The efficacy of Megamin and Immunarc *in vitro* may be due to their antioxidant, antilipidperoxidative and free radical scavenging properties that could prevent radiation-induced cell injury. Results demonstrated that Megamin acts mostly on genetic level, reducing the incidence of micronuclei, whereas Immunarc protects cells membrane. Furthermore, combination of these nutraceuticals significantly increased apoptotic index, more efficiently than single nutraceuticals. The results presented here indicate that synergistic action of investigated nutraceuticals provides better therapeutic applications. According to the results of present study, they activate appropriate DNA repair patway or, in the case of irreparable damage, induce apoptosis.

Obtained results confirmed that supplementation with both nutraceuticals can be used to mitigate adverse effects of ionizing radiation. Although mechanisms underlying their protective activities are not fully understood, according to this study they can help in the radiorecovery and radioprotection.

Conclusion

The application of nutraceuticals Megamin and Immunarc can help in prevention and treatment of injurious caused by ionizing irradiation.

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