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Evaluation of an Immunomodulator Drug as a Radioprotectant on Human Peripheral Blood Lymphocytes *In Vitro*

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Abstract

Background: IMOD™, a selenium enriched extract of the plants Tanacetum vulgare, Urtica dioica, and Rosa canina, has an excellent effect on oxidative stress. In this study, we investigated the radioprotective effects of this immunomodulatory drug on human peripheral blood lymphocytes.

Methods: Peripheral blood samples obtained from venipuncture of the brachial vein were treated with IMOD™ (5, 10, 15, 20 µl) for 30 min and Cobalt 60 γ-rays (0.25, 0.5, 1, 2 Gy) as the test groups and cultured with the control. We used the micronuclei assay, cell death detection, and cell toxicity assay to analyze the treatment effects.

Results: The frequency of micronuclei were 1.66 (0 Gy), 5.33 (0.25 Gy), 9.67 (0.5 Gy), 17.67 (1 Gy), and 23.67 (2 Gy) in the irradiated lymphocytes (P<0.001). The percentage of micronuclei frequency reduced to 20%, 26.83%, 37.68%, 16%, and 20.47% with IMOD™. Apoptosis and necrosis decreased significantly in the IMOD™ treated groups (P<0.05).

Conclusion: IMOD™ may protect these cells against ionizing radiation.

Keywords: Immunomodulator drug (IMOD™), Radioprotectant, Lymphocyte, Micronuclei assay, Apoptosis

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Introduction

It is well known that radiotherapy or radiation from natural and industrial sources can be harmful to normal human tissues.[1,2] Ionizing radiation (IR) generates reactive oxygen species (ROS) such as, H_2O_2, OH^O, and O_2. These products are highly reactive and can potentially react with cellular biomolecules such as proteins and DNA to result in various oxidative lesions.[3] It has been suggested that increased levels of ROS might be involved in cellular dysfunction and cell death, and cause DNA strand breaks.[4-6]

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Ionizing radiation activates the inflammatory process through the expression of pro-inflammatory cytokines such as interleukins-1 and -6 (IL-1 and IL-6) and mediators of cytokines such as nuclear factor-kappa B and tumor necrosis factor-alpha (TNF-α).[4,5] These cytokines have anti-apoptotic effects and promote the cell proliferation phenomena.[4,5] It has been shown that antioxidants scavenge radiation induced free radicals in cells and decrease the related inflammation process.[3] They may be potentially useful as radiation protectors.[2]

A number of studies on radioprotectors have been conducted in the laboratory setting,[7-9] but the compounds were no more applicable because of the related cell toxicity and side effects.[10]

Recent researches have focused on herbal ingredients as radioprotectors because of their decreased toxicity, reduced side effects, and easier accessibility.[11] IMOD™ (Setarud) is a recently developed herbal-derived treatment for its anti-viral, anti-inflammatory, and immunoregulatory properties. It has been found to be safe when examined for its toxicity in animals.[7]

IMOD™ is an extract of Tanacetum vulgare, Urtica dioica, and Rosa canina plant species that has been enriched with selenium. The anti-inflammatory and immunoregulatory properties attributed to the herbal compound of IMOD™ and selenium were found to be an excellent protectant against oxidative stress.[12]

Differential analysis of IMOD™ has indicated that the lectin and polysaccharide fractions of Urtica dioica (nettle) exhibit anti-viral and anti-inflammatory properties[13], whereas the extract of Tanacetum vulgare possesses anti-inflammatory properties.[14] Beta-carotene in Rosa canina modulates the increased levels of cholesterol and serum blood glucose.[15] Selenium is an essential trace element that protects cells from oxidative stress.[8] It has been suggested that a diet supplemented with selenium may reduce the risk of cancer, cardiomyopathy, and immune system disorders in humans.[16]

Previous studies on IMOD™ have demonstrated a curative effect on human auto-immune disease erosive oral lichen planus (OLP), sepsis, AIDS, and inflammatory bowel disease.[17] However; we did not find any studies on the possible radioprotective effects of IMOD™. The present study has aimed to evaluate the possible radioprotective effects of IMOD™ on human peripheral blood lymphocytes *in vitro* according to the micronuclei assay. The micronuclei assay is an effective method for evaluation of clastogenic effects.[13] It is also a reliable method to measure the protective effects of compounds.[18]

Materials and Methods

Sampling

Peripheral blood samples were obtained from a volunteer, healthy non-smoker, 25-year-old man with no history of immune booster drug consumption, chemotherapy or radiotherapy, and no acute or chronic illnesses.

Micronuclei assay

Lymphocyte cultures

The whole blood samples were cultured in RPMI 1640 culture medium (Gibco, UK) that consisted of a mixture of 10% fetal calf serum, 100 U/ml penicillin, and 100 µg/ml streptomycin. Samples were treated with 5, 10, 15, and 20 µl IMOD™ (Pars Roos, Co., Iran) for 30 min. The treatment was followed by irradiation with 0, 0.25, 0.5, 1, and 2 Gy gamma rays (Cobalt-60 teletherapy unit, Theratron 1000E, Canada) in triplicate. For the micronuclei assay, the lymphocytes were induced to mitosis by 0.1% phytohemagglutinin (Gibco, UK) and incubated at 37°C in a humidified atmosphere of 95% air and 5% CO2. Cytochalasin B (final concentration: 6 µl/ml; Sigma, Germany) was added 44 h after the incubation period and the culture was harvested at 72 h. Then, the cells were suspended in a hypotonic solution (0.75 M KCl), and fixation was instantly performed in a fixative solution that consisted of methanol and acetic acid (6:1). Fixation was performed twice. The fixed cells were dropped onto clean microscopic glass slides and dried in air. Next, they were subsequently stained with a 10% Giemsa solution (Merck, Germany). The cell spreads were scored by light

microscopy at 100× magnifications to determine the frequency of micronuclei in the cytokinesis-blocked binucleated cells. We assessed 1000 binucleated cells in each sample from the triplicate cultures.

Cell death detection

The cells were seeded at a density of 5×10^4 cells per well in a 24-well plate and then treated with IMOD™ (10, 20 µl), γ-irradiation (1, 2 Gy), and the combination of IMOD™ + γ-irradiation. After a 12-h treatment period, the cells were harvested and the ELISA assay was performed using an ELISA cell death detection kit (Roche Diagnostics GmbH, Germany) according to the manufacturer's protocol. In brief, the obtained cell lysates were centrifuged at 200 g for 10 min. We transferred 20 µl of the supernatants and 80 µl of the immune-reagent that contained anti-histone-biotin and anti-DNA-peroxidase to each well of a streptavidin-coated plate. The plate was allowed to incubate at 25ºC for 2 h. Then, the wells were washed with incubation buffer and we added 100 µl of 2,2'-Azino-bis (3-ethylbenzthiazoline-6-sulfonic acid (ABTS) solution. The reactions were stopped with ABTS stop solution and absorbance was measured with an ELISA plate reader at 405 nm and a reference wavelength of 490 nm. The results were expressed as the absorbance of the treatments relative to the control group.

MTT assay

Lymphocyte preparation

The whole blood was applied onto Ficoll-Hypaque gradients and centrifuged at 800 g for 20 min. The mononuclear cells were isolated and washed twice, then suspended in RPMI 1640 medium (105 cells/ml) that contained 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 µg/ml streptomycin.

Irradiation

The samples were irradiated with 0.25, 0.5, 1, and 2 Gy gamma rays (Cobalt-60 teletherapy unit, Theratron 1000E, Canada) at a dose rate of 63.538 Gy/min in triplicate. Next, both the irradiated and non-irradiated cells were transferred to a 96-well microplate (10^5 cells per 0.2 ml media/well) and co-cultured with phytohemagglutinin (0.1% v/v) at 37°C for 24 h.

Cell viability assay

Mononuclear cells were cultured overnight in 96-well plates at a density of 15×10^3 cells per well. Then, the cells were co-incubated with various concentrations of IMOD™ (5, 10, 15, 20 µl) for 24 h followed by irradiation (0.25, 0.5, 1, 2 Gy). The negative control was not treated. Then, for the MTT assay, the cells were exposed to 50 µl MTT (2 mg/ml) for 4 h at 37°C, and finally to 200 µl DMSO. The absorbance was measured at 570 nm using the microplate reader.

Statistical analysis

Statistical analysis was performed using Graph Pad Prism 6. All data were distributed normally. One-way ANOVA, two-way ANOVA, Tukey's HSD, and Sidak tests were used for multiple data comparisons. *P*-values <0.05 were considered statistically significant.

Results

Micronuclei frequency following treatment with IMOD™ and radiation

The frequency of micronuclei were 1.66 (0 Gy), 5.33 (0.25 Gy), 9.67 (0.5 Gy), 17.67 (1 Gy), and 23.67 (2 Gy) for the irradiated lymphocytes ($P<0.001$). We observed significant decreases in cells previously treated with IMOD™ ($P<0.001$; Table 1).

Cell death differentiation following treatment with IMOD™ and radiation

Figure 1 shows the results of the differential percentage of apoptosis (Figure 1a) and necrosis (Figure 1b) in human peripheral lymphocytes treated with IMOD™ and/or IR. In the absence of IMOD™, we observed a higher rate of radiation-induced death (mainly as apoptosis) in the lymphocytes. This was particularly observed at the 2 Gy radiation dose ($P<0.0001$). However, in the presence of IMOD™ a significant decrease in radiation-induced death occurred at the 10 µl

Table 1. Micronuclei frequency in human peripheral lymphocytes treated with IMOD™ and ionizing radiation (IR) *in vitro*.

IMOD™ concentration (µl)	Radiation dose (Gy)				
	0	**0.25**	**0.5**	**1**	**2**
0	1.66±0.57*	5.333±0.57	9.667±0.57	17.67±0.57	23.67±0.57
5	1.33±0.57	4.3±0.57	7.6±0.57	15±1.00	22±1
10	1.33±0.57	4±1	6.6±0.57	14±1.00	20±1.00
15	1.33±0.57	3.3±1.15	5.6±0.57	13±1.00	18±1.00
20	1.33±0.57	2±1.00	4.3±1.11	10.3±1.53	15.3±1.52

*Data are mean ±SD

(P<0.001) and 20 µl (P<0.0001) concentrations. The cell apoptosis percent decreased in a dose-dependent manner (Figure 1). The same results were also seen at the 1 Gy radiation dose. There was approximately 30% necrosis at the 2 Gy dose. We noted a significant decrease in necrosis at the 10 µl (P<0.001) and 20 µl (P<0.0001) IMOD™ doses. Similar results were also observed with 1 Gy irradiation (Figure 1).

Cell toxicity following treatment with IMOD™ and radiation

Figure 2 shows the viability of human peripheral lymphocytes treated with IMOD™ and/or IR. The MTT assay results showed a higher viability in cells treated with different doses of IMOD™. This effect was mainly observed at the 20 µl IMOD™ dose (Figure 2).

Discussion

In the present study, we investigated the radioprotective effects of IMOD™, as an herbal extract, on human peripheral blood lymphocytes *in vitro*. The results showed that IMOD™ treatment significantly reduced the frequency of radiation induced micronuclei in lymphocytes (Table 1).

There are a number of investigations on the radioprotective effects of various herbal and chemical compounds by the micronuclei assay. Rostami et al. have suggested that co-treatment of melatonin and vitamin C may reduce genotoxicity caused by x-ray irradiation in human lymphocytes.[2] The results of our study confirmed genotoxicity of IR, however our cell treatment with IMOD™ showed more effective protection of the lymphocytes against radiation damage, which might be related to the presence of different herbal antioxidants of the compound.

Pirayesh Islamian et al. assessed the radioprotective effects of lycopene, a polyunsaturated hydrocarbon (C40H56), on lymphocytes *in vitro*. They observed that reduced radiation induced chromosome aberration to 1.63% at the 1 Gy radiation dose and 16.6% for the 2 Gy radiation

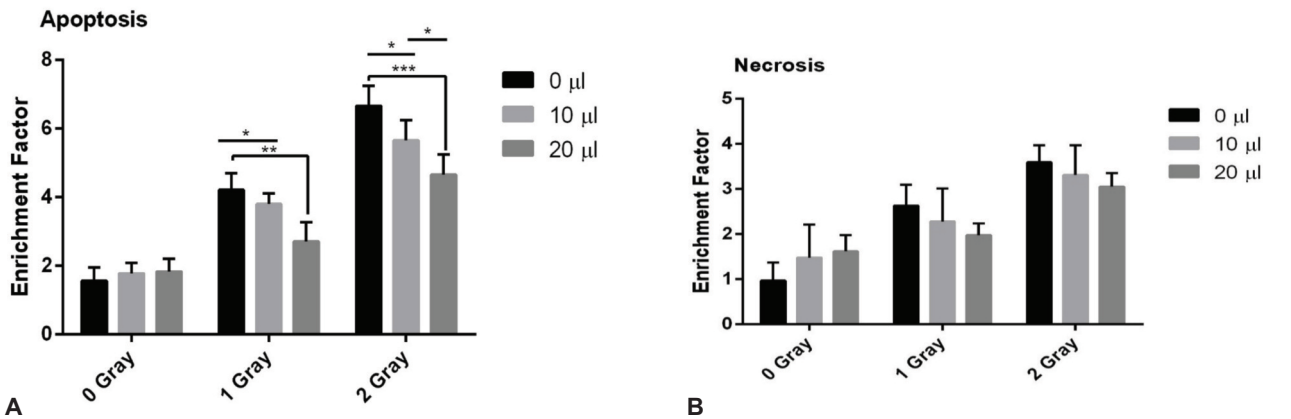

Figure 1. Differential percentage of (a) apoptosis and (b) necrosis in human peripheral lymphocytes treated with IMOD™ and/or ionizing radiation (IR). The results were expressed as mean±SD (n=3). *P<0.05, **P<0.001, ***P=0.0001.

dose.[19] Our study with IMOD™ also confirmed the radiation protection effects of herbal compounds in reducing radiation damage, as micronuclei induction, to 16% and 20.47%, respectively. Although the studied assays were different on the cells, it appeared that IMOD™ more effectively protected the cells from irradiation induced damages. This might be due to the cumulative effects of the ingredients in the herbal compound.

Ghasemnezhad Targhi et al. have reported that the Origanum vulgare leaf extract can protect mice bone marrow cells from IR damage as it is rich in powerful antioxidants, neutralize free radical activity, and reduce the secretion of nitric oxide (NO).[20] Their study has shown that gamma irradiation (3 Gy) increased the frequency of micronucleated polychromatic erythrocytes (MnPCEs), micronucleated normochromatic erythrocytes (MnNCEs), and reduced the cell proliferation ratio of polychromatic erythrocyte/polychromatic erythrocyte + normochromatic erythrocytes (PCE/PCE+NCE) in mice bone marrow compared to the non-irradiated control group (P<0.0001). Their result showed that injection of Origanum vulgare leaf extract (OVLE) significantly reduced the frequency of MnPCEs (P<0.0001) and MnNCEs (P<0.05) and increased the PCE/PCE+NCE ratio compared to the irradiated control group (P<0.05). Our study also provided confirmation of the radio-protective effect of herbals, including IMOD™, to reduce the frequency of micronuclei in human lymphocytes (P<0.05). The differences in the percentage on reduction of micronuclei might be related to the difference in experimental conditions and the radiation doses in the studies.

The radioprotective effects of Achillea millefolium L (ACM), a well-known medicinal plant called Bumadaran in Persian, against genotoxicity induced by IR in human normal lymphocytes was reported by Shahan et al.[20] They observed that the 2.5 Gy IR dose induced the percentage of micronuclei in binucleated lymphocytes (5.41) compared to untreated control lymphocytes (0.69). After pretreatment with 100 µL of ACM at the final concentrations of 10, 50, 100, or 200 µg/mL, the percentage of micronuclei in binucleated lymphocytes reduced by 1.9, 2.9, 4.8, and 7.4, respectively. In the current study, cells treated with 5, 10, 15, 20 µl IMOD™ also had reduced the frequency of micronuclei in the 2 Gy irradiated lymphocytes to 1.08, 1.18, 1.31, and 1.55, respectively. Although differences exist in the reduction factors between the studies, which should be related to the treatment doses, both studies confirmed the decrease in radiation-induced damage in cells after herbal pretreatment.

Our study showed a significant decrease in the frequency of micronuclei in the irradiated human lymphocytes pretreated with IMOD™, which was mainly observed at the 20 µl concentration. This could markedly provide reduced radiation-induced genotoxicity.

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Ethical issue

The present study did not conflict with medical ethics due to its performance as an *in vitro* study.

Formatting of funding sources

This research did not receive any specific grant from funding agencies in the public, commercial,

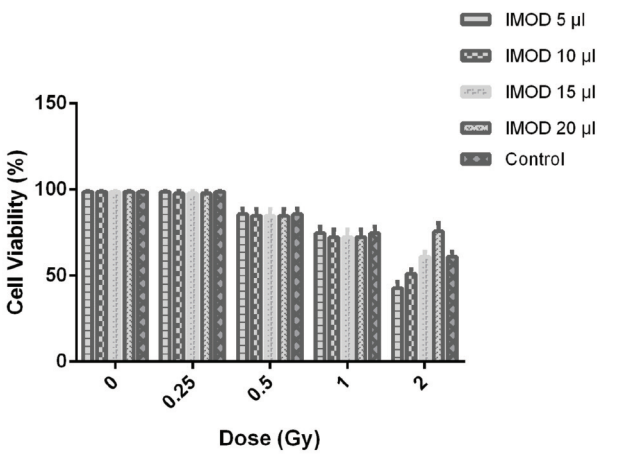

Figure 2. MTT assay results on the viability (percentage) of human peripheral lymphocytes treated with IMOD™ and/or ionizing radiation (IR).

or not-for-profit sectors.

Author contributions

All of the authors have equal contribution in the study concept and design, acquisition of data, analysis and interpretation of data, drafting of the manuscript, critical revision of the manuscript for important intellectual content, statistical analysis, administrative, technical, and material support. Jalil Pirayesh Islamian also supervised the study.

Conflicts of interests

No conflict of interest is declared.

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