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Original Contribution

RADIOPROTECTION FROM GENETIC DAMAGES BY RESURRECTION PLANT HABERLEA RHODOPENSIS - IN VIVO/IN VITRO STUDY WITH RABBITS

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ABSTRACT
The radioprotective effect of extract of resurrection plant *Habrlea rhodopensis* (HR) was investigated in cultured blood lymphocytes of rabbits. Peripheral blood samples were collected from ear marginal vein 2 hours after a single intramuscular injection of 0.03, 0.06 and 0.12 g/kg bw of *Haberlea rhodopensis* leaf extract. The whole blood samples was exposed *in vitro* to 1.0, 2.0 and 3.0 Gy of ^{60}Co gamma irradiation, and then the lymphocytes were cultured with mitogenic stimulation to determine the level of chromosome aberrations in them. The lymphocytes in the blood samples collected after extract injection exhibited a significant decrease in the incidence of metaphases containing chromosome aberrations compared to similarly irradiated lymphocytes collected from rabbits without treated the extract. A significant decrease in the frequency of aberrant cells and chromosome aberrations was observed after injection of *Haberlea rhodopensis* (0.03, 0.06 and 0.12 g/kg bw). These data suggest that it may be possible to use *Haberlea rhodopensis* extract to protect lymphocytes from genetic radiation effects.

Key words: Radioprotection; Plant extract; Gamma rays; Lymphocytes; *Haberlea rhodopensis.*

INTRODUCTION

Ionizing radiation generates reactive oxygen species (ROS) in exposed cells. These free radicals can induce damage to critical macromolecules such as DNA [1, 2]. The cellular DNA damage can then lead to mutations and cancer [3].

Chromosomal aberrations assay is frequently used to document the induction of chromosome breakage and rearrangement resulting from exposure to mutagens and carcinogens. A variety of studies have been performed to indicate that chromosome aberrations are the consequence of DNA damage and abnormal repair and replication. Radiation-induced aberrations can be observed in peripheral lymphocytes within few hours after the exposure; their frequency is related to the dose and quality of radiation and can be detected in the blood samples taken long after the exposure [4].

With respect to radiation damage to humans, it is important to protect biological systems from radiation-induced genotoxicity. One of the main radioprotective classes is thiol-based synthetic compounds such as amifostine. Amifostine is a powerful radioprotective agent compared to other agents, but this drug has limited usage in clinical setting, due to its side effects and toxicity [5, 6]. The search for less toxic radiation protectors has spurred interest in the development of natural products.

Herbal drugs offer an alternative to the synthetic compounds because they are either non-toxic or less toxic. Herbal drugs have

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recently drawn the attention of investigators. A Chinese herbal preparation from ginseng has been found to protect C3H mice against the lethal effects of γ-radiation [7]. Another herbal preparation, Liv.52, has also been reported to protect mice against radiation-induced micronucleus formation and chromosomal aberrations [8, 9].

Haberlea rhodopensis belongs to the family *Gesneriaceae* and it is a Balkan endemic relict that is widely distributed mainly in the Rhodope Mountains and some regions of the Sredna Gora Mountains and the Balkan Mountains. *Haberlea rhodopensis* belongs to the so-called"resurrection plants" due to its ability to fall in anabiosis for a long time when there are unfavourable conditions and to restore back to normal when the conditions become appropriate. *Haberlea Rhodopensis* is a relatively less explored plant. Some studies are focused mainly on its antibacterial activity [10], antioxidant potential and thermo- and photostability of photosynthesis system [11, 12, and 13] of the herb at different conditions. There are no sufficient data about the phytochemical composition in the accessible literature. A significant amount of chlorophyll and some enzymes (superoxide dismutase, citrate dehydrogenase etc.) have been found. Flavonoids, flavonoid tannin, zeaxantin, ascorbate, glutathione [14] etc. have been found in other species from the *Gesneriaceae family*.

Therefore, it was of interest to evaluate the radioprotective effect of *Haberlea rhodopensis* on radiation-induced chromosome aberrations in rabbit lymphocytes exposed to different doses of gamma irradiation.

MATERIALS AND METHODS

Preparation of drug

Leaves of *Haberlea rhodopensis* were collected from plants growing in their natural habitat (the vicinity of Bachkovo, Bulgaria) after license of Ministry of environment and water of Bulgaria. Leaves were cutted into small pieces and dried in room temperature for 1 month. After grinding the dry matter were macerated for 6 hours in 70% ethyl alcohol and were percolated for 48 h. Primary extract was concentrated by evaporation of ethanol in a vacuum environment to reach a ratio of 5% ethanol and 95% water. The obtained extract was filtered through filter paper to remove emulsified substances, chlorophyll and other particles. The extract was standardized in accordance with the method for determining the relative density (Bulgarian Pharmacopoeia Roll 2, p.19). The amount of extracted substances ranged between 0.098 and 0.113 g/cm^3 (average 0.120 g/cm^3).

Study design

In the experiment male New Zealand rabbits (5 months old, body weight 3.5-4.0 kg) were used. The animals were divided into following groups:

DDW (double distilled water) + irradiation 1Gy (n=5)
DDW (double distilled water) + irradiation 2Gy (n=5)
DDW (double distilled water) + irradiation 3Gy (n=5)
Haberlea Rhodopensis 0.03 g/kg bw+ irradiation 1Gy (n=5)
Haberlea Rhodopensis 0.06 g/kg bw+ irradiation 1Gy (n=5)
Haberlea Rhodopensis 0.12 g/kg bw+ irradiation 1Gy (n=5)
Haberlea Rhodopensis 0.03 g/kg bw+ irradiation 2Gy (n=5)
Haberlea Rhodopensis 0.06 g/kg bw+ irradiation 2Gy (n=5)
Haberlea Rhodopensis 0.12 g/kg bw+ irradiation 2Gy (n=5)
Haberlea Rhodopensis 0.03 g/kg bw+ irradiation 3Gy (n=5)
Haberlea Rhodopensis 0.06 g/kg bw+ irradiation 3Gy (n=5)
Haberlea Rhodopensis 0.12 g/kg bw+ irradiation 3Gy (n=5)

The animals were injected (IM) with HR 2 hours before taking blood. Blood samples were obtained from the marginal ear vein in sterile tubes with 30 U/mL heparin as anticoagulant.

Irradiation:

Whole blood samples were exposed to 1.0 Gy, 2.0 Gy and 3.0 Gy gamma rays ^{60}Co at a dose rate 89.18 cGy/min in a water bath (37^{0}C).The samples were kept at 37^{0}C for 1 h soon after irradiation and then transported to the laboratory.

Preparation of lymphocyte cultures

The method of Evans [15] with modification for rabbits was used. 0.5 mL of irradiated whole heparinised blood was incubated in 7 mL RPMI 1640 medium, 3 mL heat-inactivated normal calf serum, 0.2 mL reconstituted PHA, 100E/mL penicillin, and 50 μg/mL genthamicin. The

cultivation flasks were thermostated in the dark at 39^0 C. Each group included two cultivation flasks from each donor.

All cultures were incubated for 48 hours. Colcemid at a final concentration of 0.2 μg/ ml was added at 46 h to block the cells at metaphase stage. At the end of the 48^{th} hour from the beginning of lymphocyte incubation, chromosomal preparations for detection of chromosomal aberrations were prepared.

100 metaphases were scored for each rabbit. The data are presented as the number of chromosome aberrations/100 cells.

Statistics:

The $\bar{x}$±SD was calculated for each parameter. Data were analysed using ANOVA with multiple comparison test. To evaluate the protective property of extract, treated cultures (HR+radiation) were compared with the cultures exposed to gamma rays only.

RESULTS

The frequencies of aberrant cells, dicentric aberrations, acentric fragments and total aberrations (including rings and exchanges) in cultured rabbit lymphocytes irradiated to gamma rays are presented in **table 1, table 2** and **table 3.**

Exposure of blood samples to 1.0 Gy, 2.0 Gy and 3.0 Gy resulted in a dose-dependant elevation in the frequency of chromosome aberrations. Maximum number of aberrant cells (31.6±6.9) and total aberrations (52.8±10.18) has been found after exposure to 3 Gy.

All concentrations of Haberlea Rhodopensis (0.03, 0.06 and 0.12 g/kg bw) significantly ($P<0.05$) decreased the frequencies of chromosome aberrations, when the lymphocytes were exposed to all doses.

Table 1 showed that pretreatment of rabbits with extract of HR reduced the number of chromosome aberrations. The lowest frequencies of aberrant cells (4.8±1.68) and total aberrations (7.6±1.14) were observed after pretreatment with 0.12 g/kg bw HR.

Table 1. *Effect of HR pretreatment on the frequency of chromosome aberrations in lymphocytes irradiated at 1.0 Gy. (a- P<0.05; b- P<0.01; c- P<0.001 vs. the same parameter of 1.0Gy)*

Dose	Treatment	Meta-phases	Aber. cells.	Fragments	Dicentrics	Total aberr.
0	untreated	500	2.4±1.14	2.6±1.14	0	2.6±1.14
1.0 Gy	DDW	500	9.6±1.5	9.4±1.94	5.2±1.3	15.2±3.11
1.0 Gy	HR 0.03 g/kg	500	7.2±1.92 a	6.0±1.22 b	3.6±0.83 a	11.0±2.23 a
1.0 Gy	HR 0.06 g/kg	500	5.8±1.3 b	5.8±2.16 b	3.4±0.89 a	9.6±0.73 b
1.0 Gy	HR 0.12 g/kg	500	4.8±1.68 c	4.4±1.14 c	2.8±0.83 b	7.6±1.14 c

After irradiation to 2 Gy and 3 Gy the frequencies of chromosome aberrations decreased with increasing the concentration of HR. The level of aberrant cells and total aberrations after pretreatment with 0.12 g/kg bw HR was 6.6±1.12; 11.4±2.07 in lymphocytes irradiated to 2 Gy and 12.6±2.5; 23.6±4.39 in lymphocytes irradiated to 3 Gy. These results showed that there was a dose-dependant increase of chromosome aberrations in gamma irradiated lymphocytes and that the pretreatment with extract of HR reduced frequency in a dose-dependent manner.

Table 2. *Effect of HR pretreatment on the frequency of chromosome aberrations in lymphocytes irradiated at 2.0 Gy. (a- P<0.05; b- P<0.01; c- P<0.001 vs the same parameter of 2.0Gy)*

Dose	Treatment	Meta-phases	Aber. cells.	Fragments	Dicentrics	Total aberr.
2.0 Gy	DDW	500	20.6±4.66	24.2±5.44	11.6±1.14	37.6±5.5
2.0 Gy	HR 0.03 g/kg	500	14.2±1.92 a	14.6±2.96 b	9.4±1.67 a	25.2±5.26 b
2.0 Gy	HR 0.06 g/kg	500	9.0±2.0 c	9.6±3.04 c	6.25±1.39 c	16.46±3.50 c
2.0 Gy	HR 0.12 g/kg	500	6.6±1.12 c	6.2±1.92 c	4.8±1.48 c	11.4±2.07 c

Table 3. *Effect of HR pretreatment on the frequency of chromosome aberrations in lymphocytes irradiated at 3.0 Gy.(a- P<0.05; b- P<0.01; c- P<0.001 vs. the same parameter of 3.0Gy)*

Dose	Treatment	Meta-phases	Aber. cells.	Fragments	Dicentrics	Total aberr.
3.0 Gy	DDW	500	31.6±6.9	31.0±9.0	21.8±2.58	52.8±10.18
3.0 Gy	HR 0.03 g/kg	500	22.6±3.91 a	26.2±4.02	16.2±2.58 b	41.8±5.01
3.0 Gy	HR 0.06 g/kg	500	18.6±2.30 b	21.4±2.94 a	10.2±1.48 c	33.40±3.12 b
3.0 Gy	HR 0.12 g/kg	500	12.6±2.50 c	15.00±3.53 b	7.8±1.64 c	23.6±4.39 c

DISCUSSION

It is known that radiation damage of DNA and other molecules may cause gene mutations. Gene mutation in germ cells is expressed in descendants, whereas it is expressed in the individual if it occurs in somatic cells. Radiation has also been reported to produce immediate and delayed effects [16].

Scoring of chromosome aberrations allows a direct assessment of the genotoxicity of various physical and chemical agents.

Increased production of reactive oxygen species (ROS) is considered as the ultimate consequence of many environmental stresses, including radiation [17] and the main cause for lipid peroxidation, protein denaturation and DNA damage [18, 19]. The use of certain chemicals may help to reduce/inhibit the genotoxicity, which in turn may inhibit mutagenesis and carcinogenesis.

Chromosome aberrations are highly quantifiable manifestations of radiation-induced damage to DNA that may be observed in the first post-irradiation mitosis, and studies conducted in plants and animals employed scoring of chromosome aberrations as a method to quantify levels of radioprotection by various SH compounds [20].

Several botanicals have been screened for their radioprotective activity [21, 22, 23]. Oral administration of M. piperita (1g/kg body weight/day) before exposure to gamma radiation was found to be effective in protecting against the chromosomal damage in bone marrow of Swiss albino mice [24].

A similar *in vivo/in vitro* method in human blood lymphocytes was used for assessing melatonin [25] and hawthorn [26] for radioprotection.

We previously reported that extract of Haberlea rhodopensis protected cultured lymphocytes from gamma irradiation induced chromosome aberrations *in vitro* [27]. In this study the administration of HR 2 hours before irradiation of obtained blood samples reduced the level of radiation induced chromosome damages in peripheral lymphocytes.

HR was equally effective in protecting against radiation-induced chromosome damage in rabbit peripheral lymphocytes as evidenced by a reduction in the frequency of chromosome aberrations like dicentrics, acentric fragments and total aberrations.

The results of this study showed the protective effect of extract of Haberlea rhodopensis against genotoxicity induced by gamma irradiation in rabbit lymphocytes, after intra muscular injection of the extract. The exact mechanism of action of chromosome protection by HR is not known.

The radioprotective effect of plants and herbs may be mediated through several mechanisms, since they are entities of many chemicals. The majority of plants and herbs contain polyphenols, scavengers of radiation-induced free radicals and elevation of cellular antioxidants by plants and herbs in irradiated systems could be the leading mechanism for radioprotection [28].

Phenolic phytochemicals are a large group of substances ubiquitous in plants and found in significant quantities in vegetables, fruits and beverages such as teas. They have a number of biological effects *in vivo* and *in vitro* and have been regarded as possible antioxidant, anti-inflammatory, antiviral and antiallergic agents [29,30]. Some of the phenolic phytochemicals such as flavonoids have been suggested to inhibit oxidative damage and effectively protect cells and tissues against the deleterious effects of reactive species [31, 32]. However,

the full chemical properties and effects of phenolic phytochemicals have not completely been examined and it is also unclear whether all phenolic phytochemicals have beneficial effects. There is also considerable evidence that some phenolic phytochemicals are mutagenic and clastogenic in both mammalian and bacterial experimental systems [33, 34]. Phenylethanoid glycosides are a group of phenolic compounds mainly spread in several botanical families of Tubiflorae (Verbenaceae, Lamiaceae, Scrophulariaceae) [35, 36]. Plantainosides A-F, Sanangosid and Myconosid isolated from Ramonda Mycona family Gesneriaceae [37] have antioxidant property [38, 39]. Glycoside Myconosid was found in Haberlea.

Therefore, the reduction in chromosomal damage in the present study may be due to a free radical scavenging activity of HR as well as protection against radiation induced DNA damage.

CONCLUSION

Results of this simple experiment showed that without Haberlea treatments, gamma radiation significantly increased aberrant cells and chromosome aberrations frequencies in peripheral blood lymphocytes.

A protective effect was seen in chromosome aberration yields of samples irradiated with 1.0, 2.0 and 3.0 Gy and treated with *Haberlea rhodopensis* 2 h before irradiation at 0.03, 0.06 and 0.12 g/kg/bw. The data obtained in our study suggested that HR extract prevented DNA damage inflicted by gamma irradiation. Despite its limitation, this study has added new data to growing body evidence that some plant extract were effective radioprotectors. However, before their approval for clinical use, further experimental studies using at the same time different cytogenetic and molecular biomarkers and well designed clinical studies are needed to clarify the exact mechanisms of their radioprotective action and possible interactions.

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