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Evaluation of Radiomodulatory Effect of Haberlea Rhodopensis (Friv.) Extract Using Micronucleus Assay in Lymphocytes of Whole Body Irradiated Rabbits.

KEYWORDS: γ-rays; micronucleus; Haberlea rhodopensis; radioprotection

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ABSTRACT *Haberlea rhodopensis leaf extract (HR) possess strong antioxidant activity. The present study aimed to examine the radioprotective potentiality and efficacy of HR against DNA damage induced by γ-irradiation to dose range 1.0-3.0 Gy. HR total extract (0.12 g/kg b. w.) was injected im, into rabbits 2h before whole body irradiation. Rabbits were exposed to 1.0, 2.0 and 3.0 Gy 60Co γ-rays. The radiation-induced changes were estimated by using cytokinesis blocked micronucleus assay (CBMN) in peripheral lymphocytes. Results reveled that the micronucleus test pointed out a significant difference between groups received HR before irradiation and γ-irradiated groups. It was concluded that HR extract was effective in the protection against clastogenic effect of radiation due to its content of different antioxidant ingredients and their ability to scavenge free radicals generated by radiation.*

Introduction

Ionizing radiation is known to affect somatic and germ cells, leading to mutations, cell death, malformations and cancer. It is generally considered that many radiation-induced biological effects could be attributed to the activated water reaction, which produced different kinds of free radicals (19). These reactive species can induce damages to cellular macromolecules. Both direct and indirect effects of ionizing radiation damage cellular DNA. DNA lesions induced by radiation have already been suggested and it is believed that chromosomal aberrations appear as a result of double strand breaks and misrepaired damage (13). Damage to chromosomes is also manifested as breaks and fragments, which appears as micronucleus (MN) in the rapidly proliferating cells (7).

Study on plant extracts and phytochemicals as modifiers of radiation effects is a new area of research. Some reports are available regarding plant extracts and phytochemicals on radiation induced cellular damage in model as well as in animal systems (6,16).

Haberlea rhodopensis Friv. (family *Gesneriaceae*) is a rare perennial herbaceous plant native of the Balkans. HR is a poikilohydric species which is highly desiccation-tolerant and able to revive upon rehydration of vegetative tissues even after prolonged periods of complete dehydration. HR has been studied in a phytochemical survey of the family *Gesneriaceae*, whereby the occurrence of myconoside, a caffeoyl phenylethanoid glucoside, was reported (10). Flavone 8-C glycosides, flavanoids and five compounds possessing antiradical activity were identified in methanolic extracts of HR (2,3).

The aim of this study was to evaluate the radiomodulatory effect of total extract of HR in rabbits l exposed *in vivo* to γ-irradiation by using Micronucleus Assay.

Materials and methods

Preparation of drug

The total extract of HR was prepared by maceration of the plant leaves for 48 h in 70% aqueous-ethanol solution and a subsequent alcohol distillation in a vacuum evaporator until a drug/liquid phase ratio of 5:1 was reached (1 mL of the extract contained 0.12 g of extracted substances).

Study design

In the experiment male New Zealand rabbits (5 months old, body weight 3.5-4.0 kg) were used. The experimental protocol was approved by the Department of Animal Care and adhered to the European Community Guiding Principles for the Care and Use of Animals.

The animals were divided into following groups: I. Untreated (control) group; II. HR treated group (control 2); III. DDW + irradiation 1.0 Gy; IV. DDW + irradiation 2.0 Gy; V. DDW + irradiation 3.0 Gy; VI. HR + irradiation 1.0 Gy; VII. HR + irradiation 2.0 Gy; VIII. HR + irradiation 3.0 Gy .

Rabbits (III, IV, V groups) were injected (im) with double-distilled water (DDW) 2 hours before irradiation to 1.0, 2.0 and 3.0 Gy, respectively. Animals from groups VI, VII, VII were injected (im) with HR (0.12 g/kg/b.w.) 2 hours before irradiation to 1.0, 2.0 and 3.0 Gy. Rabbits from group II were injected (im) with HR (0.12 g/kg/b.w.) along with the animals from other groups. The dose of HR (0.12 g/kg/b.w.) was selected according to our previously published study (Georgieva et al., 2012). Blood samples from all groups were obtained 24 hours after irradiation, from the marginal ear vein in sterile tube with 30 U/mL heparin as anticoagulant.

Irradiation

A cobalt teletherapy unit (Rocus M, ^{60}Co) at the Inter-District Cancer Dispensary, Stara Zagora, Bulgaria, was used for irradiation. Each rabbit, in wooden container was exposed to γ-rays, at a dose rate 80,16 cGy/min.

Micronucleous assay

Micronucleus analysis was carried out on blood lymphocyte cultures stimulated with phytohemagglutinin (2.4 µg/ml). Whole blood (1 ml) was added to 9 ml RPMI-1640 medium supplemented with 15% calf serum and lymphocytes were incubated at 39ºC. Cytokinesis was blocked by the method of Fenech (4). Cytochalasin B was added to the cultures at a final concentration of 4 µg/ml 44 h after stimulation with phytohemagglutinin. Seventy-two hours after culture initiation, cells were fixed in methanol: acetic acid (3:1) after 5 min of mild hypotonic treatment (0.56% KCl + 0.9% NaCl mixed in equal volumes). Slides were air-dried and stained with 5% Giemsa.

In each group a total of 5000 (1000 cells from 5 donors) binucleated cells were scored and the frequency of cells with one (MN1), two (MN2) and three (MN3) micronuclei was recorded. The total number of MN in each group was derived from (1×MN1) + (2×MN2) + (3×MN3). The data are presented as

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the number of MN per cell.

Statistical analysis
Data were presented as mean ± SD, n=5. Statistical analysis was performed using Student's t-test. The value of $p < 0.05$ was considered as significant.

Results and discussion
The frequency of MN in rabbit lymphocytes induced by various doses of radiation and in combination with HR are shown in table 1.

The established spontaneous micronucleus frequency in the lymphocytes of untreated rabbits was 0.0084±0.0038 MN/cell. There exists no authenticated difference between the untreated rabbits and the animals treated with and HR extract.

After 1.0 Gy gamma irradiation, the binucleated cells showed only one or two MN frequencies. MN frequency was increased with increase in the dose (2 and 3 Gy) of radiation. Collectively, γ-irradiated lymphocytes showed a dose-dependent increase in the total number of MN frequencies. Pretreatment of HR with different doses of radiation showed a significant ($P< 0.05$) reduction in the MN frequencies and the protection was at the range of 48-64% in all the HR+ radiation treated groups, which is the biological index for the detection of inhibiting potential of HR on cellular toxicity and clastogenicity induced by γ-irradiation.

Because of extensive use of ionizing radiation in medicine, agriculture and industry the acquisition of radioprotectors is an urgent need for the society. However, the applicability of radioprotectors currently under investigation is limited due to their inherent toxicity.

In search for new source of radioprotective agent, the extract of HR was subjected to analysis. The micronuleus test in binucleated cells was applied as an ancillary test system to the chromosomal aberrations analysis. Irradiation of rabbits in this study led to an extreme increase in the frequency of MN, compared to the control. The increase of the MN frequency could be explained by the clastogeneic ability of γ-irradiation. The radiation-induced MN frequency has been reported by others (9,11). But, pre-treatment of rabbits with HR prior to irradiation (1.0-3.0 Gy) decreased the frequencies of MN in a dose-dependent manner. At all applied doses, HR effectively protected the lymphocytes from γ-irradiation with % of reduction ranged from 64.2 (1.0 Gy) to 48 (3.0 Gy). Earlier we showed the similar phenomenon with radiation-induced chromosomal aberrations in rabbit lymphocytes *in vitro* (15).

The present results indicate that HR might have reduced genetic damage through its anticlastogenic potential. Extract of HR contains high level of flavanoid antioxidants. Several phenolic compounds were identified, mainly phenolic acids, flavanoid aglycones and glycosides (12). The antimutagenic and radical scavenging activity of phenolic acids was reported by many authors (1,20). A number of flavanoids (genistein, quercetin, luteolin) have been found to reduce the frequency of micronucleated reticulocytes in the peripheral blood of irradiated mice (18). Protection of DNA from oxidative stress by luteolin, quercetin and rutin was demonstrated *in vitro* in the Comet assay (8). On the basis of those studies it is possible to explain the radioprotective capability of HR and resulted reduction of MN frequency through free radical scavenging activity of many compounds presented in the extract.. In addition some studies also support that HR is an antioxidant, having antibacterial, antimutagenic and immunomodulatory properties (5,14,17).

Conclusion
HR extract is effective in protection against the clastogenic effect of radiation due to its content of different antioxidant ingredients and its ability to scavenge free radicals generated by radiation. However, the specific mechanism of HR extract in anticlastogenic activity needs further investigation in the future.

Table 1. Frequency of MN in binucleated cells following irradiation to 1.0, 2.0 and 3.0 Gy and pre-treatment with an HR extract.

Groups/ Treatment	Total cells scored	Cells with MN	MN distribution MN1	MN2	MN3	MN/cell Mean ±SD	% reduction
I. Untreated	5000	42	42	-	-	0.0084±0.0038	
II. HR	5000	44	40	4	-	$0.0096±0.0017^{ABC}$	
III.1.0 Gy	5000	226	196	11	-	$0.0452±0.019^{A}$	
IV. 2.0 Gy	5000	625	452	65	11	$0.125±0.046^{B}$	
V. 3.0 Gy	5000	1215	670	202	47	$0.243±0.058^{C}$	
VI. HR+1.0 Gy	5000	81	87	7	-	$0.0162±0.08^{A}$	64.2
VII. HR+2.0 Gy	5000	225	103	49	8	$0.062±0.015^{B}$	50
VIII. HR+3.0 Gy	5000	605	363	99	22	$0.0121±0.07^{C}$	48

Identical letters showed statistical significance between groups.

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