Cellular DNA damage and lipid peroxidation after whole body gamma irradiation and treatment with *Haberlea rhodopensis* extract in rabbits

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SUMMARY

The aim of the study is to investigate radioprotective effects of total extract from *Haberlea rhodopensis* (a natural plant in the Balkan region) *in vivo*. For that, alkaline comet assay was performed on peripheral lymphocytes and plasma MDA (a marker of lipid peroxidation) concentrations were determined in New Zealand white rabbits (5 animals per group) 24 hours after whole body exposure to γ-radiation (2.0 Gy) or sham irradiation eventually associated to intramuscular injection of 0.24 g/kg *H. rhodopensis* extract 2 hours before or 30 minutes after irradiation. The comet frequency, reflecting cellular DNA damage, was dramatically increased in lymphocytes from irradiated rabbits but this increase was significantly limited (by 40%) when *H. rhodopensis* extract was administered preliminary to irradiation. In addition, plasma MDA concentrations remained low in γ-exposed rabbits treated before or after by *H. rhodopensis* extract whereas they were markedly elevated in animals only submitted to γ-radiations. The results demonstrate the direct and indirect radioprotective effects *in vivo* of *H. rhodopensis* extract probably mediated by some antioxidant compounds.

Keywords: Rabbit, comet assay, *Haberlea Rhodopensis*, DNA damage, lipid peroxidation, radioprotection, antioxidants.

RÉSUMÉ

Lésions de l'ADN cellulaire et péroxydation lipidique après irradiation γ du corps entier et traitement par un extrait d'*Haberlea rhodopensis* chez le lapin.

L'objectif de cette étude a été d'explorer les effets radioprotecteurs *in vivo* de l'extrait total d'*Haberlea Rhodopensis* (une plante de la région des Balkans). Pour ce faire, l'analyse des comètes en milieu alcalin a été réalisée sur les lymphocytes périphériques et les concentrations plasmatiques de MDA (marqueur de la péroxidation lipidique) ont été mesurées chez des lapins blancs de Nouvelle Zélande (5 animaux par groupe) 24 heures après exposition du corps entier à des radiations γ (2,0 Gy) ou à une exposition factice éventuellement associée à une injection intramusculaire de 0,24g/kg d'extrait de *H. rhodopensis* 2 heures avant ou 30 minutes après. La fréquence des comètes (réflétant des lésions de l'ADN cellulaire) a été considérablement augmentée dans les lymphocytes issus des lapins irradiés mais cette augmentation a été significativement limitée (de 40 %) lorsque l'extrait d'*H. rhodopensis* a été administré préalablement à l'irradiation. De plus, les concentrations plasmatiques de MDA sont restées relativement faibles chez les lapins irradiés et traités par l'extrait de plante avant ou après alors qu'elles ont été très nettement augmentées chez les animaux seulement exposés aux radiations γ. Ces résultats démontrent les effets radioprotecteurs directs et indirects de l'extrait d'*H. rhodopensis in vivo* probablement dus à la présence de différents antioxydants.

Mots clés : Lapin, analyse des comètes, *Haberlea Rhodopensis*, lésions de l'ADN, péroxydation lipidique, radioprotection, antioxydants.

Introduction

Ionizing radiation undoubtedly can damage DNA by directly ionizing DNA itself and by indirect processes in which DNA reacts with numerous radiolysis reactive products including $OH^{\bullet}$, $H^{\bullet}$, $O2^{\bullet-}$ and H_2O_2, that are generated in aqueous fluid surrounding DNA [28]. Investigations have shown that oxygen free radicals may cause lipid peroxidation and oxidative stress [24, 25]. Recent literature reveals enormous interest in the radioprotecting property of synthetic drugs used for treating various human disorders, as well as herbal extracts, phytoceuticals and neutraceuticals [9, 11-14, 16, 17, 27, 34].

Alcoholic extracts of *Haberlea rhodopensis*, a naturally occurring plant that is spread mainly in the Rhodope Mountains and some regions of the Sredna gora Mountains and the Balkan Mountains, were found to possess strong antioxidant and antibacterial activities [31, 32]. Flavonoids, flavonoid tannin, zeaxantin, ascorbate, glutathione and other antioxidant compounds [21] have been found in other species from the *Gesneriaceae* family. Preliminary phytochemical studies indicated that *H. rhodopensis* contains flavone C- glycosides, caffeoyil phenylethanoid glucosides [10] in

addition to previously reported lipids and saccharides [26]. Recently, BERKOV *et al.* [7] through GC-MS metabolic profiling of the apolar and polar fractions from methanolic extracts of *Haberlea rhodopensis* revealed more than one hundred compounds (amino acids, fatty acids, flavonoids and phenolic acids, sterols, glycerides, saccharides, *etc.*). Bioactivity assays showed that the polar fractions possessed strong free radical scavenging activity IC50 values (concentrations causing 50% scavenge of the $DPPH^{\bullet}$) with 19.95 ± 14.11 µg/mL for fresh leaves and 50.04 ± 23.16 µg/mL for desiccated leaves, and both the polar and apolar fractions failed to provoke any significant cytotoxic effects against the tested cell lines. Five compounds possessing antiradical activity were identified, i.e. syringic, vanillic, caffeic, dihydrocaffeic and *p*-coumaric acids. The aim of the present study is to evaluate the radioprotecting ability of total extract of *Haberlea rhodopensis*.

Materials and Methods

PREPARATION OF DRUG

The leaves of plant were dried and roughly pulverized. A total extract from *Haberlea rhodopensis* leaves macerated for 48 hours in 70% water-ethanolic solution with subsequent distillation of the ethanol in vacuum evaporizer to a drug/liquid phase proportion of 5:1, was used.

ANIMALS AND PROTOCOL DESIGN

In the experiment, 25 male New Zealand rabbits (5 months old, body weight 3.5-4.0 kg) were used and were randomly allotted into 5 equal groups of 5 rabbits in each group according to the *Haberlea rhodopensis* treatment and the irradiation dose (rabbits were placed in a ventilated plexibox and their entire bodies were exposed to a dose of 2.0 Gy (dose rate of 24 cGy/min) of ^{60}Co-γ rays delivered by a Gamma Rocus). Two control groups were submitted to sham-irradiation alone or in combination with intramuscular injection of 0.24 g/kg *H. rhodopensis* extract 30 minutes after (groups 1 and 2, respectively). Animals from the 3 other groups were γ-irradiated and received intramuscular injection of distilled water (2 mL) (group 3) or 0.24 g/kg *H. rhodopensis* extract 30 minutes after (group 4) or 2 hours before (group 5). These animals were maintained under controlled conditions of temperature and light as per norms laid down by a departmental ethical committee.

Blood samples were collected 24 hours after irradiation by puncture of the auricular vein into sterile microtubes with EDTA as anticoagulant for comet assay and MDA determination: 1 mL of each sample was transferred in a 1.5 mL testtube (Eppendorf) and was rotated at 700 g on a centrifuge for 3 minutes at 4°C. The cell sediment was suspended in phosphate buffer-saline (PBS). A volume of 75 µl of the cell suspension was mixed with 100 µl 1.4 % low-melting agarose (LMA) for mounting upon the microscopic slides. After centrifugation of whole blood samples at 1 500 g for 15 minutes at 4°C plasmas were used for determining MDA concentrations.

BIOCHEMICAL ANALYSES

Comet assay: Damage to cellular DNA was measured by alkaline comet assay [20] in peripheral blood lymphocytes. The above-described suspension of cells and agarose was spread as micro-gel upon a microscopic slide. Following the agarose polymerization, the slides with the micro-gel were dipped in a cold lysis solution (1M NaCl, 50 mM EDTA pH 8, 30 mM NaOH, 0.1% N-lauroylsarcozine pH 10) for 1 hour. DNA was denatured by incubating the slides 3 x 20 minutes in a denaturing solution (30 mM NaOH pH 12.6, 10 mM EDTA pH 8). Electrophoresis was conducted at 10°C for 20 minutes, 0.45 V/cm in an electrophoresis buffer (30 mM NaOH pH 12.6, 10 mM EDTA pH 8). The glass slides were then washed with 0.5 M Tris-HCl, pH 7.5 to neutralize the alkali and were dehydrated by sequential washes in 75 and 95% ethanol for 5 minutes each. The comets in the gel were stained with 0.5 µg/mL ethidium bromide and visualized by a fluorescent microscope. About 100 comets were scored for each rabbit. The comets were analysed by visual classification as described by JALOSZYNSKI *et al*., [18]. Damage was assigned to 5 classes (0-4) based on the visual aspect of the comets, considering the extent of DNA migration.

Plasma MDA concentrations: the thiobarbituric acid reactive substances were measured according to the method described by PLASER and CUSHMAN [29]. Briefly, 1 mL plasma, 1 mL physiological solution, and 1 mL 25% trichloracetic acid were mixed and centrifuged at 700 g for 20 minutes. Protein free supernatants (1 mL) were mixed with 0.25 mL 1% thiobarbituric acid and heated at 95°C for one hour. After cooling, the absorbance of the end fraction product (Malondialdehyde, MDA) was determined at 532 nm (A_{532}). The MDA concentration was calculated according to the following formula:

[MDA] (µmol/L) = 1.75 x A_{532}/ 0.156 (extinction coefficient: 1.56 x 10^5 M^{-1} cm^{-1}).

Statistical analysis

Results are presented as mean and standard deviation (SD). Statistical analysis was performed using Student's t-test for comparison of the differences between groups and one way ANOVA. The value of $P < 0.05$ was considered as significant.

Results

As shown in figure 1, the percentages of comets evidenced 24 hours after treatments have significantly increased in lymphocytes from rabbits treated with *H. rhodopensis* extract and/or exposed to γ-radiations compared to the group 1 (no radiation, no herbal extract). Indeed, the comet percentage increased approximately 2 times with *H. rhodopensis* extract treatment alone ($P < 0.05$) and was about 6-fold higher than that in the group 1 when rabbits were exposed to γ-radiations without extract treatment (group 3) ($P < 0.001$). In irradiated rabbits co-treated with *H. rhodopensis* extract before or after γ-exposure (groups 4 and 5, respectively), the comet frequencies were also markedly enhanced compared to the group 1

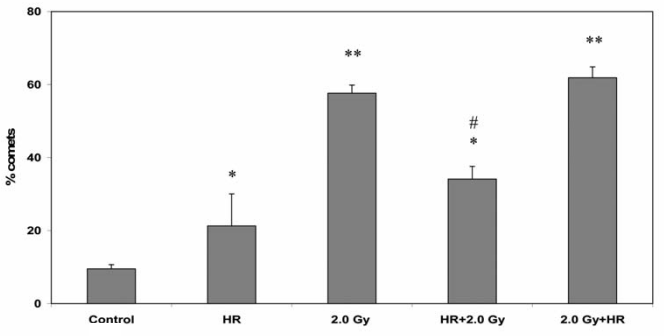

FIGURE 1 : Comet percentages in lymphocytes collected in rabbits 24 hours after γ-radiation (2.0 Gy) exposure coupled to intramuscular injection of 0.24g/kg *H. rhodopensis* (HR) extract 30 minutes after or 2 hours before whole body irradiation. Results are presented as mean ± standard deviation (SD). **P* < 0.05 and ***P* < 0.001 vs. control group; #*P* < 0.05 vs. irradiated group only.

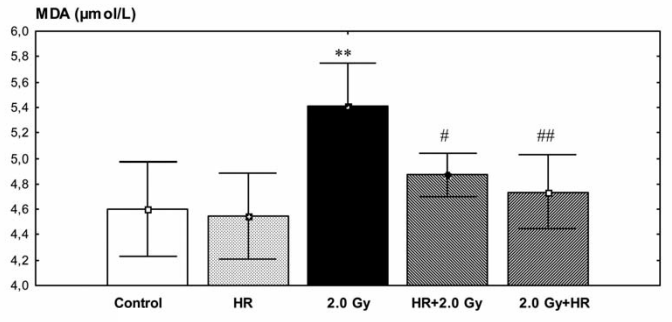

FIGURE 2 : Plasma MDA concentrations in rabbits 24 hours after γ-radiation (2.0 Gy) exposure coupled to intramuscular injection of 0.24g/kg *H. rhodopensis* (HR) extract 30 minutes after or 2 hours before whole body irradiation. Results are presented as mean ± standard deviation (SD). ***P* < 0.01 vs. control group; #*P* < 0.05 and ## *P* < 0.01 vs. irradiated group only.

(*P* < 0.05 and *P* < 0.001, respectively). However, the *H. rhodopensis* extract administration following the irradiation (group 4) has not exhibited a protective effect and the comet percentage was closely related to that found in rabbits only subjected to radiations whereas the administration of the extract before the whole body irradiation has induced a significant decrease in the comet formation by approximately 40% in comparison with the group 3 (*P* < 0.05).

Figure 2 illustrates the plasma MDA concentrations reflecting lipid peroxidation eventually induced by body irradiation and/or herbal extract injection. It was recorded that treatment with *H. rhodopensis* extract alone has induced no significant variation of the MDA concentrations while they were dramatically increased after γ-irradiation compared to the non irradiated control rabbits (*P* < 0.01). By contrast, compared to the group 3 (subjected to γ-radiations only), plasma MDA concentrations were significantly lowered by approximately 10% in rabbits exposed to γ-radiations and treated with the herbal extract 30 minutes after (*P* < 0.01) or 2 hours before (*P* < 0.05). However, no significant difference was evidenced between the 2 groups of γ-exposed rabbits treated with *H. rhodopensis* extract.

Discussion

Ionizing radiation is a potent DNA-damaging agent, throughout production of free radicals which may interact directly and indirectly on cellular DNA for inducing molecular lesions in irradiated cells. The incidence of cell cycle disturbances, aberrant mitoses or cell death may rise as the dose of irradiation increases. Starting from the nucleus, the primary target of radiation damage is DNA [4]. Apart from DNA damage, lipid peroxidation is also considered to be a critical event of ionizing radiation effect [1]. Lipid peroxidation has been found to increase with increase in radiation dose in rat liver mitochondria, microsomes and splenic lymphocytes [8, 22, 30].

Natural products have been traditionally accepted as remedies due to the popular belief that they produce few adverse side effects. Therefore, understanding of the potential beneficial or adverse influence of natural products extensively used by human population is very important to implement public health safety measures. In the present study, the preliminary injection of *H. rhodopensis* extract to γ-radiation exposure has induced a significant reduction (by 40%) of comet frequency, indicating radiation protection of DNA *in vivo*. As MDA and lipid peroxidation products are known to be mutagenic in bacterial and mammalian cells [37] and carcinogenic in rats [3], a reduction in MDA formation is quite desirable. It was also observed in the present study that *H. rhodopensis* extract administration has markedly decreased plasma MDA concentrations in rabbits exposed to radiations, showing indirect radioprotecting effects of the herbal extract *in vivo*.

The mechanism of action of *H. rhodopensis* extract remains still unknown in part because only some compounds of the herbal extract have been identified among more than one hundred molecules [7]. Among them, flavonoids, like quercetin and phenolic acids, have been assumed to exert a major contribution to radioprotective effects [2]. Some of the radioprotective mechanisms of flavonoids are scavenger potency against free radicals [15], immunological properties [6], protection against inflammatory responses, protection against foetal effects of radiation, and induction of apoptosis in cancer cells [36]. Moreover, KUNIMASA *et al.* [23] have demonstrated that flavonoids have antioxidant properties *in vitro* and *in vivo*, and BENKOVIC *et al.* [5] also reported that flavonoids protect mice against lethal effects of whole-body irradiation, and diminish primary DNA damage.

Other five compounds possessing antiradical activity, namely syringic, vanillic, caffeic, dihydrocaffeic and p-coumaric acids [33], were identified in total extract of *H. rhodopensis*. SESTILI *et al*. [35] reported that the most prominent activity of caffeic acid is that it prevents DNA single-strand breakage and cytotoxicity. It also has the ability to chelate iron. According to KALKAN *et al.* [19], concentrations of some individual phenolic constituents (syringic, p-coumaric and vanillic acids) are correlated with high antioxidant activity and inhibition of LDL oxidation. Negative correlations between vanillic acid and MDA concentrations and between p-hydroxybenzoic acid and oxidized LDL were confirmed by principal component analysis (PCA) analyses. In agreement to that, it was hypothesized that *H. rhodopensis* extract would indirectly lower lipid peroxidation and cellular DNA damage induced by radiations by limiting free radical formation be-

cause of the presence of various radical scavengers in the herbal extract.

As a conclusion, the alkaline single cell gel electrophoresis analysis of peripheral blood lymphocytes from whole body irradiated rabbits displayed the increased comet parameters indicating radiation-induced damages like formation of alkali labile sites and single and double strand breaks for example. The decrease in the comet frequency in irradiated rabbits pretreated with *H. rhodopensis* extract indicated reduction in radiation induced cellular DNA damage and evidenced some *in vivo* radioprotective effects of the herbal extract. In the same way, marked decrease in plasma MDA concentrations in irradiated rabbits treated with *H. rhodopensis* extract revealed that the extract has limited lipid peroxidation therefore contributing indirectly to the radioprotective effects *in vivo*.

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