JPP Journal of Pharmacy and Pharmacology

JPP 2005, 57: 135–143

Received May 26, 2004
Accepted October 4, 2004
DOI 10.1211/0022357055218
ISSN 0022-3573

Protection of mitochondrial system by *Hippophae rhamnoides* L. against radiation-induced oxidative damage in mice

Harish Chandra Goel, Damodar Gupta, Shobha Gupta, A. P. Garg and Madhu Bala

Abstract

The whole extract of the fresh berries of *Hippophae rhamnoides* L. (RH-3), which has been reported to provide protection to whole mice, various tissues, cells and cell organelles against lethal irradiation, was further investigated for its effects on mitochondria isolated from mouse liver. Superoxide anion, reduced (GSH) and oxidized glutathione (GSSG) levels, NADH–ubiquinone oxidoreductase (complex I), NADH–cytochrome c oxidoreductase (complex I/II), succinate–cytochrome c oxidoreductase (complex II/III), mitochondrial membrane potential (MMP), lipid peroxidation (LPx) and protein oxidation (PO) were determined for RH-3-mediated radioprotective manifestation. Pre-irradiation treatment of mice with RH-3 (30 mg $kg^{-1,}$ i.p.; single dose; −30 min) significantly inhibited the radiation-induced increase in superoxide anions, GSSG, thiobarbituric acid reactive substances (TBARS), complex I, complex I/III activity and MMP maximally at 4 h ($P < 0.05$). This treatment inhibited the oxidation of proteins ($P < 0.05$) at all the time periods studied here. This study suggests that pre-irradiation treatment of mice with RH-3 protects the functional integrity of mitochondria from radiation-induced oxidative stress.

Radiation Biology Division, Institute of Nuclear Medicine and Allied Sciences, Brig. S. K. Mazumdar Road, Delhi-110054, India

Harish Chandra Goel*, Damodar Gupta†, Madhu Bala

Department of Microbiology, Chaudhary Charan Singh University, Meerut-250005, India

Shobha Gupta, A. P. Garg

Correspondence: H. C. Goel, Department of Applied Microbiology, Chaudhary Charan Singh University, Meerut-250005, India. E-mail: goelharish@hotmail.com

Present address: *Department of Applied Microbiology, Chaudhary Charan Singh University, Meerut-250005, India. †Department of Cancer Biology, Cleveland Clinic Foundation, Research Institute, Cleveland, OH-44120, USA.

Acknowledgement and funding: This research was sponsored by Defence Research and Development organization, Ministry of Defence, Government of India. Under this research project no tests were done on human beings.

Introduction

Radiation-induced reactive oxygen species cause damage to various cellular components, including mitochondria (Somosy 2000), which undergo structural and functional changes, especially in the electron transport chain (ETC). This leads to increased leakage of electrons (Zhang et al 1990) and reduction of molecular oxygen to superoxide anions (O_2^-), which become dismutated to yield hydrogen peroxide (Cai & Jonas 1998). Excessive production of reactive oxygen species (ROS) depolarizes the mitochondrial membrane potential (MMP) and results in decreased production of adenosine triphosphate (ATP). These events also cause opening of the mitochondrial permeability transition pore (MPTP) and release of cytochrome c into the cytosol, leading to apoptotic cell death (Crompton 1999).

Under normal conditions, generation of ROS occurs at mitochondrial complex I and III via the Q-cycle. The oxidative modification of mitochondrial bio-molecules impairs ETC activity, which increases the ROS generation further via a vicious cycle. The intrinsic mitochondrial components, such as quinol, cytochrome c, vitamin E, manganese-dependent superoxide dismutase (MnSOD) and reduced glutathione (GSH), counteract the oxidative stress. However, due to the additional oxidative burden induced by radiation, mitochondria suffer bio-energetic disturbances. Therefore, extraneous supplementation of antioxidants in the cellular milieu does provide protection to mitochondria. A number of purified natural compounds, like quercetin (Yang et al 2001) and chlorophyllin (Boloor et al 2000) and extracts of plants, like *Podophyllum hexandrum* (Goel et al 1998; Gupta et al 2003), *Asparagus racemosus* (Kamat et al 2000), *Hippophae rhamnoides* (Goel et al 2002; Geetha et al 2003) and *Tinospora cordifolia* (Goel et al 2004), have been reported to scavenge free radicals, up-regulate antioxidant enzyme components and inhibit lipid peroxidation and protein oxidation to provide protection against radiation-induced damage and mortality.

H. rhamnoides L., commonly known as sea-buck-thorn (family: Elaegnaceae), growing in North-West Himalayas at high altitude, 7000–15 000 feet, has been widely used in traditional medicine for treating many diseases, like gastric ulcers (Suleyman et al 2001; Xing et al 2002), liver disorders (Cheng et al 1984), skin disorders (Yang et al 2000) and ischaemic heart (Zhang 1987), and for prevention of coronary heart disease (Eccleston et al 2002). The berries have also been used to make ready-to-serve food items. The berries contain polyphenolic compounds (viz. isorhamnetin, rhamnetin, quercetin, kaempferol), carotenes (α, β, γ), vitamins (C, E, riboflavin), folic acid, tannins, glycerides of palmitic, stearic and oleic acids, and some essential amino acids (Chan et al 1990; Chu et al 2003; Yue et al 2004). These constituents present in the whole extract manifest radioprotection by several mechanisms, like free-radical scavenging, metal chelation, chromatin compaction and hypoxia induction (Goel et al 2003; Prem Kumar et al 2002). In our laboratory the whole extract of the berries of *H. rhamnoides* (RH-3) has been shown to provide over 80% whole-body survival to mice subjected to lethal doses of γ-radiation (Goel et al 2002).

In a cell, mitochondria are the major site of oxidative stress because of some leakage of electrons during ATP production and also due to inefficient machinery for repair of mitochondrial DNA. For repair of nuclear DNA and recovery of many intracellular organelles energy is the most important requirement. In a cell mitochondria are the most important source for energy generation. Therefore, any antioxidant that manifests a radioprotective effect should also protect mitochondria. Protection of the mitochondrial system can have far reaching significance in the prevention and recovery of free-radical-induced chronic disease, such as arteriosclerosis, arthritis and several neurological disorders. Therefore this study was undertaken to investigate whether RH-3 protected mitochondria against radiation-induced oxidative stress.

Material and Methods

Chemicals and plant material

3-(4,5-Dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), *o*-pthalaldehyde (OPT), *N*-ethylmaleimide (NEM), 2-thiobarbituric acid (TBA), trichloroacetic acid (TCA), cytochrome c, rotenone, antimycin A, gramicidin, β-NADH, bovine serum albumin (BSA) and dinitro-phenyl hydrazine (DNPH) were obtained from Sigma Chemicals Inc. (St Louis, MO), while rhodamine-123 was obtained from Fluka (Switzerland) and HEPES from Calbiochem (San Diego, CA). All other chemicals utilized were of ANALAR grade and were obtained from Indian firms.

The aqueous alcoholic extract (50:50 v/v) of fruits (dried berries) of *H. rhamnoides*, collected from North-West Himalayas at high altitude, 7000–15 000 feet, in the month of April to June, was prepared as described earlier by Goel et al (2002). The yield was approximately 8.9% w/w.

Experimental animals

Male inbred Swiss Albino strain A mice, 8–10 weeks old, 25 ± 3 g, maintained under controlled environment (25 ± 3°C, 12-h light–dark cycle) were provided with free access to standard animal food pellet (Amrut Laboratory Animal Feed, India) and water. Animal experiments were conducted according to INSA-Ethical Guidelines for the use of Animals in Scientific Research published by the Central Drug Research Institute, Lucknow, India, after taking approval from the Animal Experimentation Ethics Committee of the laboratory.

Animal groups

Untreated control (n = 4) 3: 0.2 mL vehicle (i.p.) and sham irradiated.

Irradiated control (n = 4) 3: 10 Gy whole body γ-irradiation.

Drug control (n = 4) 3: 0.2 mL 30 mg kg^{-1} (body weight) in vehicle (−30 min; i.p.; single dose) before sham irradiation.

RH-3 + Irradiation (n = 4) 3: 0.2 mL 30 mg kg^{-1} (body weight) in vehicle (−30 min; i.p.; single dose) before 10 Gy whole body irradiation.

n represents the number of mice in each experiment of a treatment group; the number mentioned outside the brackets indicates number of experiments repeated.

Irradiation

Whole-body irradiation to mice was given through ^{60}Co γ chamber (Model 220–Atomic Energy of Canada) having a dose rate of 0.481 Gy min^{-1} at room temperature. Fresh air was continuously circulated in the irradiation chamber to avoid generation of hypoxic conditions.

Isolation of mitochondria

Mice were sacrificed by cervical dislocation, dissected and liver was taken out and visible blood clots were removed. Liver homogenate (10%) was prepared in ice-cold isolation medium (0.3 M sucrose, 0.1% BSA, 1 mM EGTA, 5 mM Mops, 5 mM KH_2PO_4, pH 7.4) using a Potter Elvjham homogenizer and mitochondria were isolated using the method of Rickwood et al (1987). Briefly, the homogenate was centrifuged at 1000 *g* for 10 min at 4°C. The supernatant was collected and centrifuged at 10 000 *g* to obtain the mitochondrial pellet. The mitochondrial pellet was washed three times with BSA-free isolation medium and the purity and integrity of mitochondria were identified by measuring the monoamine oxidase enzyme activity.

Mitochondria were separately isolated for measurement of lipid peroxidation. The mitochondrial pellet was washed three times with 50 mM potassium phosphate buffer (pH 7.4) to remove traces of sucrose (which also reacts with TBA).

Mitochondrial glutathione estimation

Mitochondrial glutathione was determined using the method of Hissin & Hilf (1976). Briefly, mitochondria

(1 mg protein) were suspended in 25 μL of 25% HPO_3 and 90 μL of sodium phosphate buffer (0.1 M, pH 8.0, with 5 mM EDTA). The samples were centrifuged at 15 000 *g* for 10 min at 4°C and the supernatant was collected for measurement of total glutathione (GSX) and oxidized glutathione (GSSG). Supernatant (100 μL) was incubated with 100 μL of *o*-phthalaldehyde (0.1% in methanol) and 1.8 mL of 0.1 M phosphate buffer (pH 8.0) for 15 min at room temperature in the dark. Fluorescence was measured with a fluorescence spectrophotometer (Varian, USA) at an excitation wavelength of 350 nm and an emission wavelength of 420 nm.

For determination of GSSG, 40 μL of 0.04 M *N*-ethylmaleimide was added to the supernatant and the resulting solution was incubated for 30 min at room temperature and the procedure mentioned above was adopted, except that 0.1 M NaOH was used in place of 0.1 M phosphate buffer. GSSG concentration was measured using the calibration curve.

Mitochondrial superoxide generation

Freshly isolated mitochondria (100 μg/100 μL) were placed in micro-titre plates (Corning, USA) and 6 μL of MTT solution (1.25 mM in PBS; pH 7.4) was added to each well. The plates were then incubated at 37°C for 30 min. After incubation, the formazan formed due to reduction of MTT was dissolved using 150 μL of DMSO and measured at 570 nm (Madesh & Balasubramanian 1997). The amount of superoxide generated was calculated using the molar extinction coefficient of MTT formazan (E_{570} of 17 000 $mol^{-1}\,cm^{-1}$) at pH 7.4–8.0 and the results were expressed as nmol of superoxide anion generated per minute per mg of mitochondrial protein.

Mitochondrial enzyme assays

The mitochondrial suspension was freeze-thawed, gently shaken three times to ensure mitochondrial lysis and the activity of ETC enzymes was measured at room temperature in a total reaction volume of 1 mL using a UV-Vis spectrophotometer. Each observation was carried out in triplicate.

Complex I (NADH–ubiquinone oxidoreductase)
The activity was measured by recording the decrease in absorbance due to oxidation of NADH to NAD at 340 nm, with 425 nm as a reference wavelength ($\in = 6.81\ mmol^{-1}\,cm^{-1}$; Kwong & Sohal 2000). The reaction mixture contained 25 mM potassium phosphate buffer (pH 7.4), 5 mM magnesium chloride, 2 mM potassium cyanide, 2.5 mg BSA, 100 μM ubiquinone and 2 μg antimycin A. The reaction was initiated by addition of mitochondria (50 μg of protein) and monitored for 2 min. After a measurable linear rate was observed, 5 μg of rotenone was added to the reaction mixture to obtain the rotenone-insensitive rate. The complex I activity was determined as the difference between the total enzymatic rate and that obtained with the addition of rotenone. Each observation was carried out in triplicate and was repeated three times.

Complex I/III (NADH–cytochrome c oxidoreductase)
The activity was measured by recording the increase in absorbance due to the reduction of ferricytochrome c at 550 nm, with 580 nm as the reference wavelength ($\in = 19\ mmol^{-1}\,cm^{-1}$; Kwong & Sohal 2000). The reaction was initiated by addition of 50 μg of mitochondrial protein to the assay mixture (50 mM potassium phosphate buffer (pH 7.4), 80 μM ferricytochrome c, 100 μM NADH, 5 mM magnesium chloride, 2 mM potassium cyanide) and the enzymatic activity was monitored for 1.5 min. Rotenone (5 μg) was added thereafter and the reaction was monitored for an additional 1 min. The rotenone-insensitive NADH–cytochrome c oxidoreductase rate was then calculated by subtracting the rotenone-sensitive rate from the overall rate.

Complex II/III (succinate–cytochrome c oxidoreductase)
The activity was measured by recording the increase in absorbance due to reduction of cytochrome c at 550 nm with 580 nm as the reference wavelength ($\in = 19.1\ mmol^{-1}\,cm^{-1}$; Kwong & Sohal 2000). Mitochondrial protein (50 μg) was pre-incubated with assay mixture (40 mM potassium phosphate buffer (pH 7.4), 20 mM succinate, 2 μg rotenone, 2 mM potassium cyanide and 0.5 mM EDTA) for 20 min at room temperature. Thereafter, the reaction was initiated by the addition of 30 μM cytochrome c and monitored for 2 min.

Mitochondrial membrane potential

Mitochondrial membrane potential (MMP) was measured using the method of Emaus et al (1986). Freshly isolated mitochondria were incubated in respiration buffer (0.22 M sucrose, 68 mM mannitol, 10 mM potassium chloride, 5 mM potassium dihydrogen phosphate, 2 mM magnesium chloride, 500 μM EGTA, 5 μM succinate, 2 μM rotenone, 10 mM HEPES, pH 7.2) supplemented with 0.5 μM rhodamine-123 (Rh-123) for 5 min. Changes in the fluorescence of Rh-123 were measured at 25°C using a fluorescence spectrophotometer (Varian, USA) at an excitation wavelength of 490 nm and an emission wavelength of 532 nm. Gramicidin (20 μM) was used for abolishing the mitochondrial membrane potential and to measure non-specific uptake of Rh-123.

Mitochondrial lipid peroxidation

Thiobarbituric acid reactive substances (TBARS) were measured spectrophotometrically as described by Buege & Aust (1991). Briefly, mitochondrial protein (4 mg mL^{-1}) was mixed with an equal volume of Buege & Aust reagent (TCA, 15% (w/v) in 0.25 M HCl; TBA, 0.37% (w/v) in 0.25 M HCl) and heated for 15 min in boiling water. After cooling, the precipitate was removed by centrifugation at 1000 *g* in a table-top refrigerated centrifuge (Kendro Instruments, USA) for 10 min at room temperature. The absorbance of the supernatant was recorded at 532 nm spectrophotometrically against a sample containing reagents but no sample. The concentration of TBARS was determined using an extinction coefficient of $1.56 \times 10^5\ mol^{-1}\,cm^{-1}$ and

results expressed as nmol of MDA per mg of mitochondrial protein.

Mitochondrial protein oxidation

Oxidative damage to mitochondrial protein was measured as protein carbonyl formation or amount of DNPH incorporated per mg of miochondrial protein (Schild et al 1997). Mitochondrial protein was precipitated with ice-cold TCA (20% final concentration) and redissolved in 300 μL of 0.1 M sodium hydroxide. Three samples were treated with 0.3% DNPH (prepared in 2 M HCl) and one with HCl alone (for background reading). Both samples were kept in the dark at room temperature for 1 h and the samples were shaken every 15 min. The reaction was stopped by addition of TCA (20%) and the samples were centrifuged at 1000 *g* for 10 min. The pellet was washed three times with ethanol–ethyl acetate (1:1) and once using TCA (20%). The washing step involved resuspension of the pellet followed by recentrifugation. To evaporate the residual organic solvent, the pellet was incubated at 37°C for 15 min under nitrogen flux. Afterwards, the pellet was dissolved in 6 M guanidine hydrochloride (prepared in a mixture of HCl and acetate buffer, pH 2.3). The difference in absorbance between DNPH-treated and HCl-added samples was determined at 370 nm spectrophotometrically. Protein recovery was estimated in each sample. The calculations were done using a molar extinction coefficient of aliphatic hydrazones (22 000 $mol^{-1}\,cm^{-1}$ for the DNPH derivatives) and results were expressed as nmol of protein carbonyl per mg of mitochondrial protein.

The protein concentration in each sample was measured by the method of Lowry et al (1951) using BSA as standard.

Data analysis

Data with respect to different parameters obtained from mice belonging to various treatment groups were subjected to multivariate two-way analysis of variance using SPSS software and the significance was assessed at 95% confidence level. Bonferroni and LSD method was used for post-hoc comparison between the treatments and also between the different time intervals at 95% confidence level.

Results and Discussion

Mitochondrial glutathione

Irradiation maximally increased the levels of reduced glutathione (GSH) at 1 h, though the levels were significantly high with respect to untreated control at 4 and 8 h ($P < 0.05$; Table 1). Administration of RH-3 to mice also

Table 1 Effect of RH-3 (30 mg kg^{-1}, administered to mice i.p., −30 min) on various biochemical parameters and components of mitochondria; changes recorded as fold increase or decrease with respect to untreated control

Biochemical parameter/component	Group	1 h	2 h	4 h	8 h	24 h
GSH (nmol (mg mitochondrial protein)$^{-1}$)	10 Gy	5.12	1.28	2.26	2.60	1.61
	RH-3	3.69	1.65	2.15	1.50	1.77
	RH-3 + 10 Gy	4.75	1.85	2.14	3.37	2.08
GSSG (nmol mg mitochondrial protein)$^{-1}$)	10 Gy	2.53	1.53	2.44	2.03	1.68
	RH-3	1.92	1.68	2.33	1.82	1.67
	RH-3 + 10 Gy	2.76	2.08	1.02	1.97	1.84
Superoxide (nmol min^{-1} (mg mitochondrial protein)$^{-1}$)	10 Gy	1.24	1.35	1.14	1.16	1.47
	RH-3	0.95	1.14	1.18	1.36	1.08
	RH-3 + 10 Gy	1.17	0.82	0.80	1.27	1.17
Complex I activity	10 Gy	1.78	2.05	2.74	1.62	0.85
	RH-3	0.64	0.57	3.08	1.58	0.77
	RH-3 + 10 Gy	1.12	0.65	1.36	1.36	0.68
Complex II/III activity	10 Gy	1.14	1.18	1.22	1.23	0.71
	RH-3	0.94	1.04	1.28	1.09	0.85
	RH-3 + 10 Gy	1.30	1.07	1.17	1.30	0.87
Complex I/III activity	10 Gy	1.28	2.08	3.33	0.41	0.94
	RH-3	1.73	2.23	2.26	1.15	0.80
	RH-3 + 10 Gy	3.20	2.00	2.04	2.49	0.94
Mitochondrial membrane potential	10 Gy	1.81	2.7	3.86	1.4	0.73
	RH-3	1.12	1.31	1.26	1.17	0.98
	RH-3 + 10 Gy	1.43	1.39	1.33	1.27	1.21
MDA (nmol (mg mitochondrial protein)$^{-1}$)	10 Gy	1.78	1.51	1.63	1.42	1.21
	RH-3	1.27	1.50	1.23	1.26	0.80
	RH-3 + 10 Gy	1.58	1.56	1.23	1.45	0.85
Protein carbonyls (nmol (mg mitochondrial protein)$^{-1}$)	10 Gy	1.26	1.24	1.31	1.23	1.14
	RH-3	1.08	1.17	1.04	1.05	0.98
	RH-3 + 10 Gy	1.18	1.17	1.16	1.16	1.01

significantly increased GSH levels up to 4 h with respect to untreated control ($P < 0.05$). Pre-irradiation administration of RH-3 to mice enhanced the level of mitochondrial GSH maximally at 1 h, though it remained significantly high up to 24 h with respect to untreated control and at 2 h and 8 h with respect to irradiated control ($P < 0.05$).

With respect to untreated control, irradiation significantly enhanced the oxidized glutathione (GSSG) levels at all time periods studied (up to 24 h; Table 1). RH-3 treatment significantly also increased GSSG levels up to 24 h, as compared with untreated control ($P < 0.05$). Pre-irradiation administration of RH-3 showed significantly lower levels of GSSG only at 4 h with respect to irradiated control ($P < 0.05$).

In a cell, the increase in glutathione is a cellular response to overcome the oxidative damage caused by γ-radiation (Biaglow & Varnes 1983). Following RH-3 treatment alone GSH and GSSG levels increased with respect to untreated control, which indicated the neosynthesis of glutathione. Polyphenolic compounds, like quercetin, isorhamnetin and rhamnetin, present in *H. rhamnoides* are known to elevate the levels of glutathione by up-regulating the synthesis of the enzyme γ-glutamyl cysteine synthetase (Middleton et al 2000; Myhrstad et al 2002). Pre-irradiation treatment of mice with RH-3 also resulted in enhanced levels of glutathione, and consequently GSSG, at most of the time periods studied. This could be largely responsible for mitigating the radiation-induced oxidative stress. The significant decrease in GSSG at 4 h in the RH-3-treated irradiated group in comparison with the irradiated group needs further investigation. However, it is likely that the antioxidant activity of RH-3 was two pronged, firstly stimulating the GSH production and secondly by direct scavenging of free radicals. At 4 h the RH-3 metabolites that may be responsible for direct scavenging of free radicals were maximally available. It may also be seen that at 4 h the effect of pre-irradiation treatment of RH-3 is explicitly evident on several biochemical parameters (Table 1). It may also be recalled that the antioxidative response of such molecules is dependent on the load of oxidative species. In RH-3 treatment alone, the oxidative species load is lower and, therefore, the manifestation of antioxidative action was not prominent.

Mitochondrial superoxide generation

Whole-body irradiation of mice (10 Gy) significantly increased the generation of mitochondrial superoxide anions up to 24 h as compared with untreated control (Figure 1; $P < 0.05$). Administration of RH-3 to mice did not alter the generation of superoxide anions as compared with untreated control (Table 1) and was, therefore, non-toxic. However, pre-irradiation administration of RH-3 to mice significantly inhibited the radiation-induced generation of superoxide anions at 2, 4 and 24 h ($P < 0.05$).

In a cell, mitochondria consume over 85% of molecular oxygen, which makes them more susceptible to the radiation-induced oxidative stress. Under normal physiological conditions, the reactive oxygen intermediates

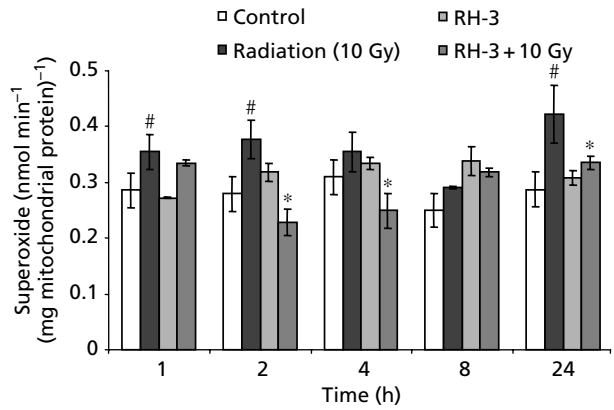

Figure 1 Effect of RH-3 (30 mg kg^{-1}; −30 min) on radiation-induced mitochondrial superoxide anion generation in mice, expressed as nmol of superoxide anion generated per minute per mg of mitochondrial protein. Data represent mean values ± s.d. of three experiments. $^{\#}P < 0.05$, untreated control vs radiation control; $^{*}P < 0.05$, radiation control vs RH-3 + radiation.

(ROI) are continuously produced in mitochondria. The generation of ROS occurs at mitochondrial complex I and complex III via the Q-cycle. The concentration of ROI is greatly enhanced following irradiation (Somosy 2000). The radiation-induced increase in leakage of electrons from the ETC complexes (complex I and complex I/III; Table 1) could have been responsible for this. The intrinsic mitochondrial components such as such as quinol, cytochrome c, vitamin E, and reduced glutathione counteract the oxidative stress. The increased levels of glutathione in RH-3-treated irradiated mice (Table 1) could have contributed to the observed decrease in the levels of superoxide anions by increased scavenging of free radicals.

Activity of complex I, I/III and II/III

Oxidative modification of ETC components or lipids impairs the transportation of electrons between ETC complexes (Konings et al 1979; Zhang et al 1990), therefore the radiation-induced change(s) in flow of electrons (complex I/III; complex II/III; Figure 2) and its modulation by RH-3 were measured. Radiation significantly enhanced complex I activity up to 8 h with respect to untreated control ($P < 0.05$), although maximum enhancement was observed at 4 h (Figure 2). The repair of radiation-induced damage required energy, which could be available by enhanced complex I activity. Administration of RH-3 to mice significantly lowered complex I activity up to 2 h with respect to untreated control ($P < 0.05$). The decrease in complex I activity at 2 h (with respect to untreated control) corroborated with the decreased GSH levels (Table 1). The decrease in GSH levels negatively affects the complex I activity (Jha et al 2000). Polyphenolic compounds are known to inhibit mitochondrial functions (Hodnic et al 1998; Middleton et al 2000). The elevation of complex I activity with increasing time of treatment with RH-3 corroborated well with increased GSH levels at observed

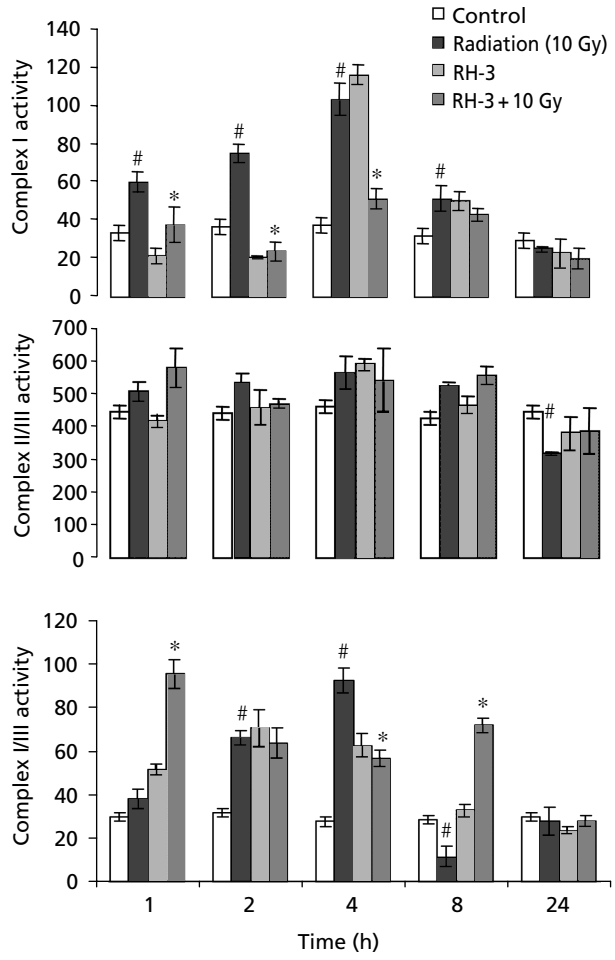

Figure 2 Effect of pre-irradiation administration of RH-3 ($30\,mg\,kg^{-1}$; -30 min) on radiation-induced changes in complex I, complex II/III and complex I/III activity expressed as nmol per minute per mg of mitochondrial protein. Data represent mean values ± s.d. of three experiments. $^{\#}P < 0.05$, untreated control vs radiation control; $^{*}P < 0.01$, radiation control vs RH-3 + radiation.

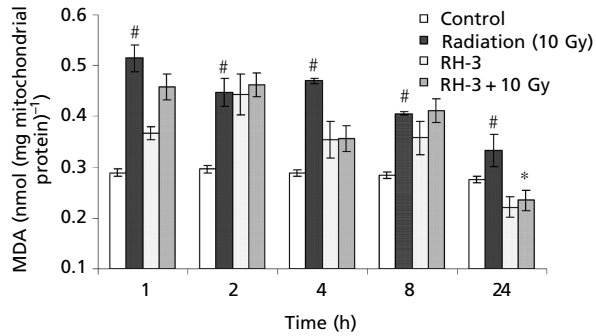

Figure 3 Protective effect of pre-irradiation administration of RH-3 against radiation-induced oxidative stress expressed as nmol of TBARS per mg of mitochondrial protein. Each value represents the mean ± s.d. of three experiments. $^{\#}P < 0.05$, untreated control vs radiation control; $^{*}P < 0.05$, radiation control vs RH-3 + radiation.

corresponding time intervals (Table 1). At 4 h there was a significant increase in complex I activity ($P < 0.05$), which tended to become normal by 24 h. RH-3 by itself has been known to act as a pro-oxidant (Goel et al 2002), a property that may be attributed to the presence of several flavonoids, as has been shown with extracts of several other plants, like *Phodophyllum hexandrum* (Prem Kumar & Goel 2000). The significant increase in the complex I activity at 4 h can be correlated with the pro-oxidative action of RH-3. This increase is corroborated by an increase in other parameters, such as superoxide anions, at 2, 4, and 8 h (Figure 1) and MDA formation at 2 h (Figure 3).

Pre-irradiation administration of RH-3 to mice significantly inhibited the radiation-induced increase in complex I activity up to 4 h ($P < 0.05$) and thereafter the activity approached untreated control levels. The significant decrease in complex I activity at 4 h ($P < 0.05$) in RH-3-treated irradiated samples could be due to the antioxidant action of RH-3. Thus RH-3 alone acts as a pro-oxidant but when the load of oxidative species exceeds a certain limit it acts as an antioxidant and acts as scavenger of free radicals. Radiation exposure certainly generates a high flux of oxidative species, which warrants antioxidant action of RH-3. It may be reiterated that a number of plant products exhibit such dual mode of action (Prem Kumar & Goel 2000).

With respect to untreated control, irradiation (10 Gy) significantly increased the complex I/III activity at 2 and 4 h ($P < 0.05$; Figure 2), which could be the result of increased activity of complex I (Figure 2). The increased generation of superoxide anions (Figure 1) and oxidative damage to lipids and proteins (Table 1) at these time intervals also indicated the interruption in the flow of electrons from complex I/III by irradiation. Radiation-mediated significant decrease in complex I/III activity at 8 h (with respect to untreated control; $P < 0.05$) can be partially attributed to the oxidative modification of lipids and proteins (Table 1). This is also correlated with a sudden drop in the MMP (Table 1). Administration of RH-3 to mice significantly enhanced the complex I/III activity up to 4 h with respect to untreated control, which can be explained in terms of the observed increase in mitochondrial activity, as also occurs during oxidative metabolism or detoxification of drugs (Nogueira et al 2001). The increase in complex I/III activity at 1 h after combined treatment (RH-3 + 10 Gy radiation) could be attributed to the excessive increase in load of oxidative species, which could be due to the combined effect of RH-3 and irradiation. A similar effect on GSH/GSSG levels has also been observed in the RH-3-treated irradiated mice at 1 h (Table 1). The protective action of RH-3 on complex I/III is envisaged maximally at 4 h when the decrease in the complex I/III activity in RH-3 pre-treated irradiated mice is significant. However, the increase in complex I/III activity at 8 h after the combined treatment (RH-3 + 10 Gy radiation) is not explainable with the present available data and more investigations are necessary to ascertain the reason. RH-3 treatment to mice

significantly ($P < 0.05$) enhanced the complex II/III activity at 4 h only (Figure 2), which is corroborated by the increase in MMP at corresponding time intervals (Table 1). Pre-irradiation administration of RH-3 to mice inhibited the radiation-mediated increase in enzyme activity at 4 h but the inhibition was not significant. This radioprotective manifestation was associated with the presence of a high amount of glutathione at 4 h (Table 1).

Mitochondrial membrane potential

The changes in MMP indicate the alteration in mitochondrial activity that may be result of physiological requirement or the effect of stressing agent. Radiation exposure enhanced MMP steeply up to 4 h and followed a decline thereafter (Table 1). The radiation-induced increase in MMP was associated with increased activity of complex I and complex I/III (Figure 2). Ionizing radiation has been reported to cause a transient increase in mitochondrial metabolism, which is decreased subsequently (Furuta et al 2004). Similarly, other agents, like He–Ne laser (Passarella et al 1984), or mild hyperthermia at a temperature of 41°C (Mikkelsen & Asher 1990), have been reported to induce increased mitochondrial function. The mechanism of such enhancement has been reported to involve up-regulation of certain genes (Gong et al 1998). However, down-regulation of mitochondrial cytochrome c oxidase by UVB radiation in human melanocytes has been reported (Crawford et al 1997; Vogt et al 1997). The increase in MMP, as observed up to 4 h in our study, could be associated with sub-lethal damage repair similar to that described in an earlier report (Hall 1994). It is presumed that whole-body exposure of mice to a lethal dose would have resulted in heterologous damage to the intracellular bio-macromolecules, which may range across lethal damage, potentially lethal damage and sub-lethal damage. The up-regulation of mitochondrial activity could be to support the activity of genes up-regulated to repair these damages.

Administration of RH-3 also enhanced the MMP up to 4 h with respect to untreated control, indicating an increase in mitochondrial activity. Pre-irradiation administration of RH-3 significantly inhibited the radiation-induced changes in MMP up to 8 h ($P < 0.05$). The decrease in MMP by pre-irradiation administration of RH-3 is possible due to decreased leakage of electrons from complex I/III and complex II/III (Table 1, Figure 2). These changes could be due to decreased generation of superoxide anions (Figure 1). Stabilizing the superoxide MnSOD has been reported to inhibit radiation-induced apoptosis in haematopoietic cells (Epperly et al 2002).

Lipid peroxidation

Exposure of mice to γ-radiation steeply increased formation of TBARS within 1 h and thereafter it decreased up to 24 h (Figure 3). However, even at 24 h the level remained significantly higher with respect to untreated control ($P < 0.05$). Increased generation of superoxide anions (Figure 1) or leakage of electrons from complex I/III and complex II/III (Figure 2) could be responsible for the increased TBARS. Administration of RH-3 also significantly enhanced the formation of TBARS up to 2 h, which can be associated with the increased mitochondrial activity, as evidenced by increased MMP at corresponding time intervals (Table 1). Pre-irradiation administration of RH-3 significantly inhibited the radiation-induced enhancement of TBARS at 4 and 24 h ($P < 0.05$), which correlated well with the high concentrations of glutathione (Table 1), decreased leakage of electrons from ETC components and scavenging of superoxide anions (Figure 1). The bioactive constituents of RH-3 like quercetin have been reported to elevate the synthesis of glutathione and also neutralize ROS (Middleton et al 2000; Yang et al 2001).

Protein oxidation

Radiation-induced increase in protein carbonyl formation was found to be maximum at 4 h and remained significantly higher up to 24 h with respect to untreated control ($P < 0.05$; Figure 4). Radiation damage to lipids (Figure 3), increased leakage of electrons from ETC complex and subsequently the increased generation of superoxide anions (Figure 1) can explain the increased formation of protein carbonyls. Administration of RH-3 also increased the formation of protein carbonyls up to 2 h, which could be attributed to increased mitochondrial activity as was also obtained by lipid peroxidation (Figure 3). Pre-irradiation administration of RH-3 to mice significantly ($P < 0.05$) inhibited the radiation-induced increase in protein carbonyls at all time periods studied. The decreased levels of superoxide anions (Figure 1), presence of high amounts of glutathione (Table 1) or free radical scavenging constituents of RH-3 explain the inhibition of radiation-induced protein carbonyl formation. The antioxidant and free-radical-scavenging potential of RH-3 has already been reported by Goel et al (2003).

Conclusion

The results of this investigation suggest that RH-3 conferred protection to mitochondria in-vivo against

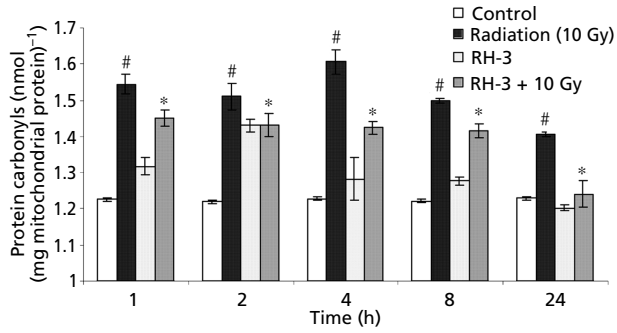

Figure 4 Effect of RH-3 on radiation-induced oxidative damage to mitochondrial proteins expressed as nmol of protein carbonyls per mg of mitochondrial protein. Data represent mean values ± s.d. of three experiments. $^{\#}P < 0.05$, untreated control vs radiation control; $^{*}P < 0.05$, radiation control vs RH-3 + radiation.

radiation-induced oxidative stress and therefore could be helpful in the prevention and treatment of several free-radical ailments associated with oxidative stress, including ionizing radiation-induced damage.

References

Biaglow, J. E., Varnes, M. E. (1983) Role of thiols in cellular response to radiation and drugs. *Radiat. Res.* **95**: 437–455

Boloor, K. K., Kamat, J. P., Devasagayam, T. P. (2000) Chlorophyllin as a protector of mitochondrial membranes against gamma-radiation and photosensitization. *Toxicology* **155**: 63–71

Buege, J. A., Aust, S. D. (1991) In: Burdon, R. H., Knippenberg, P. H. V. (eds) *Laboratory techniques in biochemistry and molecular biology*. Elsevier, Amsterdam, p. 148.

Cai, J., Jonas, D. A. (1998) Superoxide in apoptosis. *J. Biol. Chem.* **273**: 11401–11404

Chan, Y., Jiang, Z., Qin, W., Ni, M., Li, X., He, Y. (1990) Chemical composition and characteristics of sea buckthorn fruit and its oil. *Chem. Indus. Forest. Prod. (Chinese)* **10**: 163–175

Cheng, T. J., Pusk, J. K., Wu, L. W., Ma, Z. R., Cao, Z., Li, T.J. (1984) A preliminary study on hepato-protective action of seed oil of Hippophae rhamnoides L. (HR) and mechanism of action. *Zhongguo. Zhang. Yao. Za. Zhi.* **19**: 367–370

Chu, Q. C., Qu, W. Q., Peng, Y. Y., Cao, Q. H., Ye, J. N. (2003) Determination of flavonoides in *Hippophae rhamnoides* L. and its phytopharmaceuticals by capillary electrophoresis with electrochemical detection. *Chromatographia* **58**: 67–71

Crawford, D. R., Lauzon, R. J., Wang, Y., Mazurkiewicz, J. E., Schools, G. P., Davies, K. J. (1997). !6S mitochondrial ribosomal RNA degradation is associated with apoptosis. *Free Radic. Biol. Med.* **22**: 1295–1300

Crompton, M. (1999) The mitochondrial permeability transition pore and its role in cell death. *Biochem. J.* **341**: 233–249

Eccleston, C., Baoru, Y., Tahvonen, R., Kallio, H., Rimbach, G. H., Minihane, A. M. (2002) Effects of antioxidant-rich juice (sea buckthorn) on risk factors for coronary heart disease in humans. *J. Nutr. Biochem.* **13**: 346–354

Emaus, R. K., Gurnwald, R., Lemasters, J. J. (1986) Rhodamine 123 as a probe of transmembrane potential in isolated rat liver mitochondria: Spectral and metabolic properties. *Biochem. Biophys. Acta* **850**: 436–448

Epperly, M. W., Sikora, C. A., DeFilippi, S. J., Gretton, J. A. Zhan, Q. K., Donald, W., Greenberger J. S. (2002) Manganese superoxide dismutase (SOD2) inhibits radiation induced apoptosis by stabilization of the mitochondrial membrane. *Radiat. Res.* **157**: 568–577

Furuta M., Nozzaki, M., Kawashima, M., Iimuro, M., Okayama, A., Fukushima, M., Natasui, S., Souma, R., Jinnai, M. (2004) Monitoring mitochondrial metabolisms in irradiated human cancer cells with ^{99m}Tc-MIBI. *Cancer Lett.* **212**: 105–111

Geetha, S., Sai Ram, M., Singh, V., Ilavazhagan, G., Sawhney, R. C. (2003) Evaluation of antioxidant activity of leaf extract of sea buckthorn (*Hippophae rhamnoides* L.) on chromium (VI) induced oxidative stress in albino rats. *J. Ethnopharmacol.* **87**: 247–251

Goel, H. C., Prasad, J., Sharma, A., Singh, B. (1998) Antitumor and radioprotective action of *Podophyllum hexandrum*. *Indian J. Exp. Biol.* **36**: 583–587

Goel, H. C., Prasad, J., Singh, S., Sagar, R., Prem Kumar, I., Sinha, A. K. (2002) Radioprotection by a herbal preparation of *Hippophae rhamnoides*, RH-3, against whole body lethal irradiation in mice. *Phytomedicine* **9**: 15–25

Goel, H. C., Prem Kumar, I., Samanta, N., Rana, S. V. S. (2003) Induction of DNA-protein cross-links by *Hippophae rhamnoides*: implication in radiation protection and cytotoxicity. *Mol. Cell. Biochem.* **245**: 57–67

Goel, H. C., Prasad, J., Singh, S., Sagar, R. K., Agarwala, P. K., Sinha, A. K., Dogra, R. (2004) Radioprotective potential of a herbal extract of *Tinospora cordifolia*. *J. Radiat. Res.* **45**: 61–68

Gong, B., Chen, Q., Almasan, A. (1998) Ionizing radiation stimulates mitochondrial gene expression and activity. *Radiat. Res.* **150**: 505–512

Gupta, D., Arora, R., Garg, A. P., Goel, H. C. (2003) Radioprotection of HepG2 cells by *Podophyllum hexandrum* Royale. *Mol. Cell. Biochem.* **250**: 27–40

Hall, E. J. (1994) *Radiobiology for the radiobiologists*. Fourth edn, J. B. Lippincott Co., Philadelphia, PA, pp 107–132

Hissin, P. J., Hilf, R. (1976) A fluorometric method for determination of oxidized and reduced glutathione in tissues. *Ann. Biochem.* **74**: 214–226

Hodnic, W. F., Pardini, R. J. (1998) Inhibition of mitochondrial functions by flavonoids. In: Rice-Evans, C. A., Packer, L. (eds) *Flavonoids in health and disease*. Marcel Dekker, Inc., NY, pp 179–197

Jha, N., Jurma, O., Lalli, G., Liu, Y., Pettus, E. H., Greenamyre, J. T., Liu, R. M., Forman, H. J., Andersen, J. K. (2000) Glutathione depletion in PC12 results in selective inhibition of mitochondrial complex I activity. *J. Biol. Chem.* **275**: 26096–26101

Kamat, J. P., Boloor, K. K., Devasagayam, T. P., Venkatachalam, S. R. (2000) Antioxidant properties of Asparagus racemosus against damage induced by gamma-radiation in rat liver mitochondria. *J. Ethnopharmacol.* **71**: 425–435

Konings, A. W. T., Drijver, E. B. (1979) Radiation effects on membranes I. vitamin E deficiency and lipid peroxidation. *Radiat. Res.* **80**: 494–501

Kwong, K., Sohal, R. S. (2000) Age-related changes in activities of mitochondrial electron transport complexes in various tissues of the mouse. *Arch. Biochem. Biophys.* **307**: 16–22

Lowry, O. H., Rosebrough, N. J., Farr, A. L., Randall, R. J. (1951) Protein measurement with Folin phenol reagent. *J. Biol. Chem.* **193**: 265–275

Madesh, M., Balasubramanian, K. A. (1997) A microtiter plate assay for superoxide using MTT reduction method. *Indian J. Biochem. Biophys.* **34**: 535–539

Middleton, E., Kandaswamy, C., Theoharidies, T. C. (2000) The effects of plant flavonoids on mammalian cells: implications for inflammation, heart disease, and cancer. *Pharmacol. Rev.* **52**: 673–751

Mikkelsen, R. B., Asher C. R. (1990) Effects of hyperthermia on the membrane potential and Na^+ transport of V79 fibroblasts. *J. Cell Physiol.* **144**: 216–221

Myhrstad, M. C., Carlsen, H., Nordstrom, O., Blomhoff, R., Moskaug, J. J. O. (2002) Flavonoids increase the intracellular glutathione level by trans-activation of gamma-glutamylcysteine synthetase catalytical subunit promoter. *Free Radic. Biol. Med.* **32**: 386–393

Nogueira, V., Rigoulet, M., Piquet, M. A., Devin, A., Fontaine, E., Leverve, X. M. (2001) Mitochondrial respiratory chain adjustment to cellular energy demand. *J. Biol. Chem.* **276**: 46104–46110

Passarella, S., Casamassima, E., Molinari, S., Pastore, D., Quagliariello, E., Catalano, I. M., Cingolani, A. (1984) Increase of proton electrochemical potential and ATP synthesis in rat liver mitochondria irradiated in vitro by Helium-neon laser. *Fed. Eur. Biochem. Soc. Lett.* **175**: 95–99

Prem Kumar, I., Goel, H. C. (2000) Iron chelation ad related properties of P. hexandrum, a possible role in radioprotection. *Indian J. Exp. Biol.* **39**: 1002–1006

Prem Kumar, I., Samanta, N., Goel, H. C. (2002) Modulation of chromatin organization by RH-3, a preparation of *Hippophae rhamnoides*, a possible role in radioprotection. *Mol. Cell. Biochem.* **238**: 1–9

Rickwood, D., Wilson, M. T., Darley-Usmar, V. M. (1987) Isolation and characteristics of intact mitochondria. In: Darley-Usmar, V. M., Rickwood, D., Wilson, M. T. (eds) *Mitochondria: a practical approach.* I.R.L. Press, Washington, DC, p. 4

Schild, L., Reinheckel, T., Wiswedel, I., Augustin, W. (1997) Short-term impairment of energy production in isolated rat liver mitochondria by hypoxia/reoxygenation: involvement of oxidative protein modification. *Biochem. J.* **328**: 205–210

Somosy, Z. (2000) Radiation response of cell organelles. *Micron* **31**: 165–181

Suleyman, H., Demirezer, L. O., Buyukokuroglu, M. E., Akcay, M. F., Gepdiremen, A., Banoglu, Z. N., Gocer. F. (2001) Antiulcerogenic effect of *Hippophe rhamnoides* L. *Phytother. Res.* **15**: 625–627

Vogt, T. M., Welsh, J., Stolz, W., Kullmann, F., Jung, B., Landthaler, M., McClelland, M. (1997) RNA fingerprinting displays UVB-specific disruptions of transcriptional control in human melanocytes. *Cancer Res.* **57**: 3554–3561

Xing, F., Yang, B., Dong, Y., Wang, B., Wang, J., Kallio, H. P. (2002) Effect of sea buckthorn (*Hippophae rhamnoides* L.) seed and pulp oil on experimental models of gastric ulcer in rats. *Fitoterapia* **73**: 644–650

Yang, B., Kalimo, K. O., Tahvonen, R. I., Mattila, L. M., Katajisto, J. K., Kallio, H. P. (2000) Effects of dietary supplementation with sea buckthorn (*Hippophae rhamnoides*) seed and pulp oils on the fatty acid composition of skin glycerophospholipids of patients with atopic dermatitis. *J. Nutr. Biochem.* **11**: 338–340

Yang, B., Kotani, A., Arai, K., Kusu, F. (2001) Estimation of the antioxidant activities of flavonoids from their oxidation potentials. *Ann. Sci.* **17**: 599–604

Yue, M. E., Jiang, T. F., Shi, Y. P. (2004) Fast determination of flavonoides in *Hippophae rhamnoides* and its medicinal preparation by capillary zone electrophoresis using demethyl-B-cyclodextrin and modifier. *Talanta* **62**: 695–699

Zhang, M. S. (1987) A control trial of flavonoids of Hippophae rhamnoides L. in treating ischemic heart diseases. *Chung. Hua. Shsin. Hsueh. Kuan. Ping. Tsa. Chih.* **15**: 97–99

Zhang, Y., Marcillant, O., Giulivi, C., Ernster, L., Davis, K. J. A. (1990) The oxidative inactivation of mitochondrial electron transport chain components and ATPase. *J. Biol. Chem.* **265**: 16330–16336