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Enhancement of radiation-induced apoptosis by *Podophyllum hexandrum*

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Abstract

The aqueous extract of *Podophyllum hexandrum* (RP-1), which has been recently reported to manifest radioprotective and anti-tumour properties, has been investigated for its mode of action. RP-1, under in-vitro conditions dose-dependently chelated metal ions, inhibited radiation or metal ion-induced hydroxyl radicals and lipid peroxidation and scavenged superoxide anions. Intraperitoneal administration of RP-1 to mice pre-irradiation (10 Gy) induced more DNA fragmentation and lipid peroxidation in thymocytes maximally at 4 and 8 h, respectively, in comparison with RP-1 treatment or irradiation. Flow-cytometric quantification of sub-diploid peak, oligonucleosomal cleavage assay (ladder) and depletion of total thiols also corroborated the ability of RP-1 to enhance radiation-induced apoptosis. RP-1 in presence of 100 μM $CuSO_4$ induced strand breaks in plasmid DNA and addition of metal chelators (EDTA and deferoxamine) inhibited the strand scission. Treatment with a major constituent of RP-1, podophyllin, did not cause strand breaks, but isolated constituents of RP-1, quercetin or podophyllotoxin, induced strand breaks. Depending on its concentration in the milieu, RP-1 acted as a pro- or antioxidant modifying the radiation-induced apoptosis and therefore could be exploited for cancer management.

Introduction

A cell maintains a dynamic balance between various pro-oxidant and antioxidant activities and radiation-induced free radicals are known to influence the balance towards pro-oxidants leading to oxidative stress and cytotoxicity. Irradiation increases the levels of redox reactive iron ions (Stevens et al 2000), which participate in the Fenton reaction generating reactive oxygen species like hydroxyl radicals, which can interact with different bio-macromolecules and inflict severe oxidative damage and cell death (Miller et al 1990). Under normal conditions a cell operates an effective indirect antioxidant defence system (ferritin, transferrin, ceruloplasmin etc.), which strongly binds with iron ions forbidding them from participating in reactive oxygen species generating cascade (Pal 1994). Under such conditions any agent that can bind these free and redox reactive metal ions can reduce generation of reactive oxygen species and can therefore be effective as a radioprotector. A number of agents known to have metal chelating ability are often used to control oxidative stress generated during several diseased conditions including chemotherapy and radiotherapy (Diar-Citin & Koren 1997). However, the toxicity of such agents at their useful concentration has been an important constraint (Hider et al 1994).

Radiation protection at whole organism level is a multifactorial phenomenon involving several mechanisms. For this reason singular compounds of synthetic or natural origin that have been investigated so far have not yielded a dose that is at a clinically acceptable level. The combination modality employing compounds having different modes of action has been more successful due to less toxicity and more effectiveness at lower concentrations. Plant extracts offer a natural combination of a plethora of compounds, which act through different mechanisms, which may be synergistic (Tiwari 2001). Indeed, in the Ayurvedic system of medicine such a holistic approach exploiting the synergy of various bioactive molecules in natural combinations has been exploited to cure a wide variety of ailments (Tiwari 2001).

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A number of herbal extracts have been reported to render radioprotection under in-vivo and in-vitro conditions (Goel et al 1998, 2002; Satoshi et al 2000; Prem Kumar & Goel, 2000). Podophyllum extract and its isolated constituents have been held responsible for denaturation of microtubules during metaphase. However, the role of apoptosis in radiation-induced damage and recovery has not been well established. Therefore, the role of apoptosis was investigated during this study. Cells damaged beyond repair and recovery need to be removed and replaced by new cells. This warrants accelerated apoptosis and phagocytosis of the damaged cells. Therefore, we have investigated whether radiation-induced apoptosis was modulated by the aqueous extract of *Podophyllum hexandrum* (RP-1), to ultimately facilitate manifestation of a radioprotective effect. Efforts were made to investigate three major components of *P. hexandrum* (podophyllin, podophyllotoxin and quercetin) for induction of strand breaks and apoptosis.

Materials and Methods

Chemicals

Agarose, sodium lauryl sulfate, sodium dodecyl sulfate, 5,5,-dithiobis-2-nitrobenzoic acid (DTNB), nitroblue tetrazolium, phenazine methosulfate, thiobarbituric acid, ethidium bromide, tert butyl hydroperoxide (*t*B-OOH), Triton-X-100, proteinase-K, quercetin, and Rnase were from Sigma Chemical Co. (MO). Podophyllin and podophyllotoxin were from Acoras (India). Propidium iodide was from Molecular Probes (US) and pBR322 was from Bangalore Genei. Other chemicals used were of standard make and purity.

Plant material and extract preparation

Dried rhizome of *Podophyllum hexandrum* (Field Research Laboratory, Leh, J&K, India) was powdered mechanically, mixed in 100 mL g^{-1} distilled water, incubated at 37 °C for 24 h and filtered through Whatman No. 1. The filtrate was passed through a 0.22-μm Millipore filter, lyophilized and stored at 4 °C. The stored preparation was suitably diluted in triple distilled water as per the experimental requirements.

UV-vis spectroscopy and HPLC profile of RP-1

The absorbance of RP-1 in UV and visible range was monitored in 100% methanol using a Chemito spectrophotometer. An HPLC profile of the RP-1 preparation was obtained using a C-18 Bonda pack silica column (reverse phase; Shimadzu Class VP system) and a mobile phase of methanol:water (70:30 v/v). In order to avoid variations in the bioactivity of RP-1 preparations, extracts prepared from different lots were compared with a standard RP-1 extract which had rendered 80% survival.

Irradiation

For in-vitro studies different solutions were delivered radiation doses (Gamma chamber 5000, BRIT, India) as per the requirement of the experiment. The dose rate during the course of the experiment was approximately 1.78 Gy s^{-1}. Whole body irradiation was given through a ^{60}Co gamma cell (model 220, Atomic energy of Canada Ltd) having a dose rate of 0.009 Gy s^{-1}, and fresh air was continuously circulated in the irradiation chamber to avoid hypoxic conditions. Mice kept in perforated plastic bottles were irradiated individually.

Ex-vivo/in-vitro studies

Preparation of liver homogenate and thymocytes

Randomly selected 6–8-week-old strain A male mice were killed by cervical dislocation, dissected and the abdominal cavity was perfused with 0.9% saline. Whole liver was removed and visible clots were carefully and maximally removed. A weighed amount of liver was processed to get a 10% homogenate in cold phosphate buffer saline, pH 7.4, using a Potter Elvehjem homogenizer and filtered through a nylon gauze to obtain a clear homogenate. From the same animals thymic lobes were removed, and cleaned carefully by removing clots and extraneous material. The lobes were then placed on frosted slides, minced gently and the resulting cell suspension was passed through a 25-gauge needle to avoid cell aggregates. The thymocytes were resuspended in standard buffer saline and used immediately.

Antioxidant activity

All measurements were taken on a Chemito 2500 UV-vis spectrophotometer. Estimations were expressed as inhibition (%) with respect to control, which was considered as zero inhibition. Total protein level of 10% homogenate was estimated by the method of Lowry et al (1952).

Lipid peroxidation and reactive oxygen species

The desired amount of RP-1 was added to 2 mL liver homogenate (10% w/v) in each 35-mm diam. Petri dish. Ferrous ammonium sulfate (0.5 mM 100 μL) was added to each Petri dish being incubated at 37 °C for 0.5 h followed by measurement of TBARS (Beuge & Aust 1978). Scavenging of radiation or Fenton reaction-generated OH radicals or chemically-generated superoxide anions was evaluated using 2-deoxyribose degradation and nitroblue tetrazolium reduction as marker assays (Poonam et al 1984; Gutteridge 1988).

DNA damage assays

Comet assay or strand breaks in plasmid DNA

DNA strand breaks in individual cells were detected using single cell gel electrophoresis (Comet assay) (Singh et al 1994) with minor modifications. DNA strand breaks were studied in plasmid DNA (pBR322) following the procedure described by Thiobodeau et al (2001).

In-vivo studies

Animals

Strain A Swiss albino male mice (8–12-weeks-old, 25 ± 3 g) were maintained under controlled laboratory conditions (25 ± 2 °C, 12 h photoperiod, 60–70% relative humidity). The animals were given standard animal feed (Lipton India Ltd) and tap water was freely available. Animals were used in accordance with the guidelines laid down by the Institutional Animal Ethics Committee of INMAS, Delhi, India. Animal experiments were conducted according to 'INSA-Ethical Guidelines for Use of Animals in Scientific Research', published by Central Drug Research Institute, Lucknow, India.

Treatment groups

Animals were randomly divided into four groups and killed at 0, 1, 2, 4 or 8 h after the different treatments. Group 1 ($n = 5 \times 3$) received 0.2 mL water (i.p.) and were sham irradiated. Group 2 ($n = 5 \times 3$) received 0.2 mL (200 mg kg^{-1} b.w.) RP-1 in water 2 h before sham irradiation. Group 3 ($n = 5 \times 3$) received 10 Gy whole body gamma irradiation. Group 4 ($n = 5 \times 3$) received 0.2 mL (200 mg kg^{-1} b.w.) RP-1 in water 2 h before 10 Gy whole body gamma irradiation.

DNA fragmentation assays

Agarose gel electrophoresis (ladder assay) of nuclear DNA isolated from the thymocytes was carried out following the standard methods (Ramakrishnan et al 1993). Quantitative estimation of apoptosis-induced DNA fragmentation was evaluated following the standard method reported by Ramakrishnan et al (1993). The hypodiploid or pre G1 cell population was estimated flow-cytometrically (Becton Dickenson) (Yang et al 2000).

Total thiol content and lipid peroxidation

Trichloroacetic acid (25%, 0.1 mL) was added to 0.5 mL crude thymus homogenate and, after 10-min incubation at 4 °C, it was centrifuged at 500 rev min^{-1} for 10 min at 4 °C. A 0.1-mL sample of supernatant was added to 0.9 mL 0.1 M phosphate buffer (pH 8.0) and 2 mL 0.6 mM DTNB. The absorbance was taken at 412 nm against blank which contained 0.1 mL 5% trichloroacetic acid in lieu of supernatant. The sulfhydryl content was calculated using the extinction coefficient for the complex of 136.00 mM^{-1} (Yang et al 2000). Peroxidation of lipids in thymus extract was estimated as previously mentioned for liver homogenate (Beuge & Aust 1978).

Statistical analysis

The data from three separate estimations was pooled and analysed. Significance was tested using Student's *t*-test; probability level of $< 5\%$ was considered significant.

Results

HPLC profile

The HPLC profile of RP-1 showed the presence of at least nine different constituents (Figure 1).

In-vitro antioxidant potential

OH radical or superoxide anion scavenging or lipid peroxidation

Irradiation (100 Gy) or addition of Fenton reagent (ferric chloride and ascorbic acid) considerably degraded 2-deoxyribose in solution and induced formation of thiobarbituric acid reactive substances (TBARS). However, addition of RP-1 concentration-dependently inhibited the degradation of 2-deoxyribose solution and the effect was maximum (60 or 75%) at a concentration of 1.5 or 2.0 mg mL^{-1} for irradiated and Fenton reagent groups, respectively (Figure 2). The superoxide anion generated by phenazine methosulfate and NADH significantly reduced the nitroblue tetrazolium solution. RP-1, in a concentration-dependent manner, inhibited chemically-generated superoxide anion-mediated nitroblue tetrazolium reduction (Figure 2), which was maximally (80%) effective at a concentration of 2.0 mg mL^{-1}. Incubation of liver homogenate with Fenton reagent resulted in a considerably increased amount of lipid peroxidation and formation of TBARS. RP-1 treatment concentration-dependently inhibited formation of TBARS and maximal yield (89%) was observed with a concentration of 2.0 mg mL^{-1} (Figure 2).

Metal chelation

Gel electrophoresis of thymocyte DNA (Figure 3) rendered an average tail length of 6.25 ± 0.83 μm. Tert butyl hydroperoxide (*t*B-OOH; 200 μM) induced severe strand breaks in the DNA of mice thymocytes rendering a tail length of 33.9 ± 0.76 μm. RP-1 concentration-dependently inhibited *t*B-OOH-induced strand breaks as revealed by the gradual shortening of the length of the comet tail. RP-1 (500 μg mL^{-1}) maintained the length of comet tail almost comparable with the control (8.5 ± 0.7 μm). However, butylated hydroxy toulene (100 μM), a known free radical scavenger, did not protect the DNA from

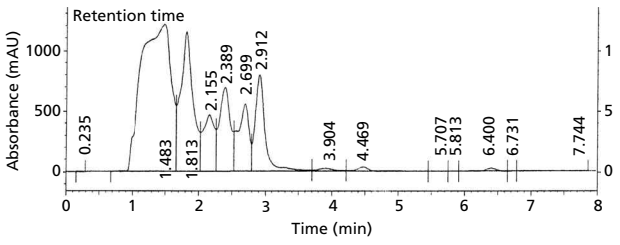

Figure 1 HPLC profile of RP-1. RP-1 was dissolved in methanol: water (70:30%) and subjected to HPLC using methanol and water (70:30%) as mobile phase.

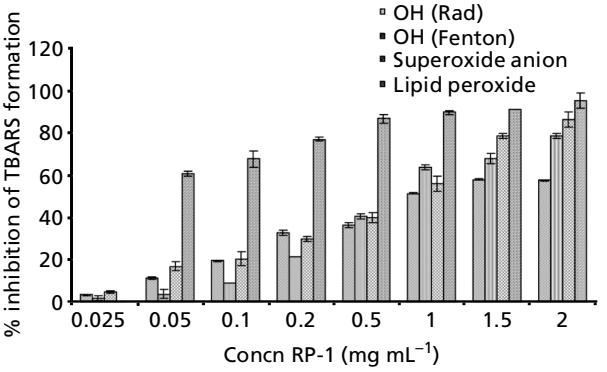

Figure 2 Effect of RP-1 on scavenging of radiation- and Fenton reaction-generated hydroxyl radicals, chemically-generated superoxide anions and lipid peroxidation. Effect of varied concentration of RP-1 on radiation or Fenton reaction-induced OH radicals and degradation of 2-deoxyribose measured as % inhibition of TBARS formation with respect to control value. Superoxide anion scavenging ability of RP-1 was estimated as % inhibition of nitroblue tetrazolium reduction (chromogen) which was calculated with respect to control (considered as zero inhibition). Each value is an average of three experiments ± s.d. All values with respect to control were significant at $P < 0.01$. x-axis in the figure is not the scale.

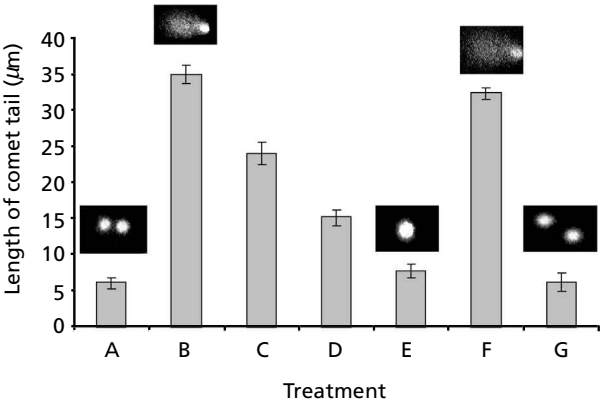

Figure 3 Effect of RP-1 on tert butyl hydroperoxide (*t*B-OOH)-induced strand breaks in thymocytes. Effect of treatment with various agents on the length of the comet tail. A. Cells without any treatment. B. *t*B-OOH (200 μM). C. 100 μg mL^{-1} RP-1 + *t*B-OOH. D. 200 μg mL^{-1} RP-1 + *t*B-OOH. E. 500 μg mL^{-1} RP-1 + *t*B-OOH. F. 200 μM BHT + *t*B-OOH. G. 25 μM 1-10 Phenanthroline + *t*B-OOH.

*t*B-OOH-induced strand breaks, comet tail length being 31.2 ± 0.6 μm. However, 1-10 phenanthroline, a standard metal chelator, completely inhibited the DNA strand breaks (6.3 ± 0.7 μm).

In-vitro pro-oxidant potential

Induction of strand breaks in plasmid DNA

In the presence of 100 μM copper sulfate, RP-1 induced strand breaks in plasmid DNA (Figure 4). RP-1 at lower concentrations (< 100 μg mL^{-1}) induced predominantly single strand breaks, but at higher concentrations double

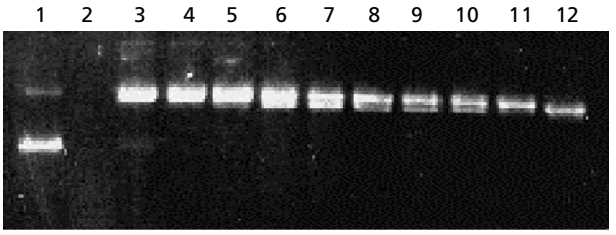

Figure 4 Induction of strand breaks in plasmid DNA by RP-1. Effect of varied concentrations of RP-1 on induction of strand breaks and conversion of fast migrating supercoiled form plasmid DNA to slow migrating relaxed form. Lane 1, control (pBR322 1 μg); lane 2, pBR322 + 50 μM $CuSO_4$ + 100 μM ascorbic acid; lanes 3–12, pBR322 + 50 μM $CuSO_4$ + 10, 20, 50, 100, 200, 300, 400, 600, 800 and 1000 μg mL^{-1} RP-1.

strand breaks were more frequent, as was seen by the appearance of a band in between the supercoiled and relaxed bands (Figure 4). The maximal concentration of RP-1 attempted here (2.0 mg mL^{-1}) converted almost the whole supercoiled DNA into the linear form. Ascorbic acid in the presence of 100 μM Cu^{2+}, used as a positive control, completely degraded pBR322 DNA forming a band at the lower part of the agarose gel. RP-1 or 100 μM copper sulfate alone under the present conditions did not induce significant strand breaks.

To test whether RP-1 induced strand breaks by generating free radicals, the influence of different antioxidants was studied. Mannitol or glycerol (standard free radical scavengers) did not inhibit the induction of strand breaks and formation of the relaxed or linear forms. However, dimethylsulfoxide, another well known free radical scavenger, was able to inhibit the strand breaks partially as was evident from the reappearance of supercoiled DNA. The standard metal chelators, EDTA (10 mM) and deferoxamine (10 mM) effectively blocked the induction of strand breaks in supercoiled DNA. Podophyllin (podophyllum resin) could not induce strand breaks at any of the concentrations tried, as can be seen from the intact supercoiled DNA form (Figure 5, lanes 9–14). However, podophyllotoxin concentration-dependently induced single strand breaks and formed an increasing amount of relaxed form, and maximum effect was observed at a concentration of 500 μg mL^{-1} (Figure 5, lanes 3–7). The highest concentration (1 mg mL^{-1}) tried in this study could inhibit strand breaks, as can be seen from the intact supercoiled DNA (lane 8). Quercetin at 25 μg mL^{-1} induced a significant amount of single strand breaks (lane 15) but it was inhibited at higher concentrations. None of the three compounds studied could induce double strand breaks (Figure 5, lanes 4–20).

In-vivo studies

Effect of RP-1 on radiation-induced apoptosis

Exposure of mice to 10 Gy gamma radiation resulted in a time-dependent increase in DNA fragments and a maximum yield (50%) was observed after 4 h irradiation, and thereafter there was a sharp decline (8 h). Mice killed at 0 h

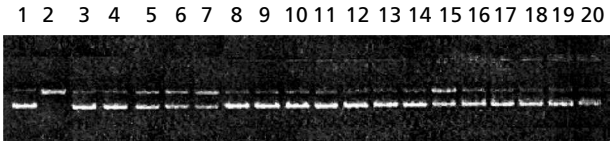

Figure 5 Podophyllotoxin, podophyllin or quercetin and DNA strand breaks in plasmid DNA. Effect of varied concentrations of podophyllotoxin, podophyllin and quercetin on induction of strand breaks in presence of 100 μM $CuSO_4$ in pBR322. Lane 1, control (pBR322 1 μg); lane 2, pBR322 + $CuSO_4$ + 25 $\mu g\,mL^{-1}$ of RP-1; lanes 3–8, pBR322 + $CuSO_4$ + 25, 50, 100, 200, 500 or 1000 $\mu g\,mL^{-1}$ podophyllotoxin; lanes 9–14, pBR322 + $CuSO_4$ + 25, 50, 100, 200, 500 or 1000 $\mu g\,mL^{-1}$ podophyllin; lanes 15–20, 1 μg pBR322 + $CuSO_4$ + 25, 50, 100, 200, 500 or 1000 $\mu g\,mL^{-1}$ quercetin.

after treatment with RP-1 (200 mg kg^{-1}) showed a significant increase in DNA fragmentation, which declined thereafter with time (Figure 6). Similarly, mice treated with 200 mg kg^{-1} b.w. RP-1 before exposure to 10 Gy gamma radiation showed a significant DNA fragmentation at 0 h compared with 10 Gy or RP-1 treatment alone. However, the frequency of fragments declined at 1 h but thereafter increased time-dependently achieving the peak at 4 h. However, at later time points the levels of fragmentation sharply declined.

Oligonucleosomal cleavage assay

During this study at 0 h oligonucleosomal ladder formation was not seen in any group. However, mice exposed to 10 Gy began showing a time-dependent increase in the amount of low molecular weight DNA, which revealed ladder formation maximally at 4 h. Mice treated with RP-1 alone did not reveal any increase in the ladder formation up to 4 h, but significantly increased after 8 h of treatment. However, RP-1-treated 10 Gy irradiation showed maximum ladder formation after 4 h irradiation but at the later time point (8 h) declined appreciably.

Quantification of sub-diploid peak

At 0 h none of the groups studied revealed induction of apoptosis, which was evident from the lack of sub-G1 peak. The 10 Gy dose induced increasingly shrunken thymocyte population in a time-dependent manner and was maximum at 4 h. RP-1 treatment alone up to 4 h did not reveal any increase in the induction of apoptosis, which significantly increased after 8 h of treatment. RP-1-treatment before 10 Gy irradiation increased the quantum of sub-diploid cell population revealing synergistic enhancement of radiation-induced apoptosis by RP-1, maximally at the 4 h time interval but declining at later time periods.

Effect of RP-1 treatment on total thiol content and lipid peroxidation in the thymocytes

Thymus was observed to have an increase in the amount of total thiol content at 0 h in all experimental groups as compared with control (Figure 7). At 4 h the levels decreased in all experimental groups. The RP-1-treated mice, with and without radiation (10 Gy), showed a recovery after 4 h but in the irradiated group % thiol continued to remain low, even up to 8 h. Different experimental groups studied revealed a decrease in the levels of lipid peroxidation up to 4 h but at the 8 h interval mice exposed to 10 Gy with or without RP-1 treatment exhibited increased lipid peroxidation compared with control.

Discussion

This study explicitly demonstrated that neutralization of OH radicals and superoxide anions was responsible for the radioprotective ability of RP-1 (Figure 2). *P. hexandrum* contains several antioxidants such as flavonoids and other polyphenolic compounds (Bors et al 1990; Singh & Shah 1994; Middleton et al 2000) and thus contributes towards radioprotection. To understand the other modes of action of RP-1 we probed its metal ion chelation activity. *t*B-OOH is known to induce DNA strand breaks in the presence of

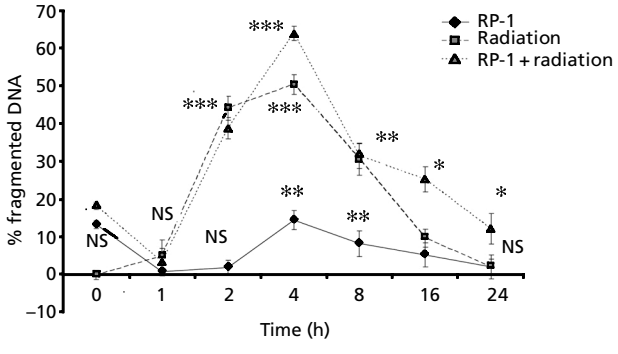

Figure 6 Induction of apoptosis in thymocytes by RP-1. Effect of varied treatments on induction of apoptosis as studied by quantifying the DNA fragmentation and expressed as % DNA fragmentation with respect to control (which was considered as 0% fragmentation). Results are mean ± s.d. of three experiments ($P < 0.05$) and compared with control.

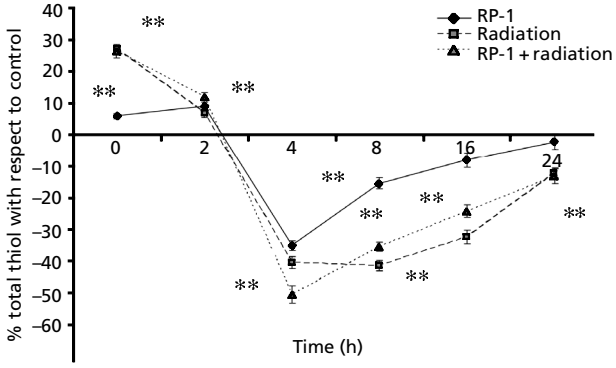

Figure 7 Depletion of cellular thiols by RP-1. Effect of different treatments on total thiols in-vivo quantified using Ellman reagent (DTNB) and expressed as % change with respect to control (which was considered as 0). Results are mean ± s.d. of three experiments ($P < 0.05$) and compared with control.

free Fe^{2+} and this reaction can be inhibited by metal chelators only and not by free radical scavengers (Sestil et al 1998). Since RP-1 inhibited *t*B-OOH-induced strand breaks, its metal chelating ability was undisputedly revealed (Figure 3).

The influence of RP-1 on radiation-induced apoptosis was investigated. Thymocytes were used for this study due to their high sensitivity to ionizing radiation and their poor DNA repair capabilities (Smith & Stark 1994). The apoptotic observations were depicted beyond 2 h treatment because the up-regulation of the apoptotic mechanism takes some time (Ramakrishnan et al 1993). RP-1 treatment alone at its radioprotective dose (200 mg kg^{-1} b.w.) and irradiation (10 Gy) enhanced apoptosis maximally at 4 h (Figure 6). RP-1 treatment before irradiation acted in an additive manner, enhancing apoptosis further. Enhanced apoptosis induced long-term augmentation of T-cell-mediated immune response (Matsubara et al 2000) and reduced mutagenic effects by removal of damaged cells (Warters et al 1997). These phenomena contribute towards radioprotection. Enhancement of apoptosis is associated with the depletion of thiols (Yang et al 2000), which was observed in all experimental groups at 4 h and beyond (Figure 7). Increased DNA fragmentation and ladder formation with corresponding depletion of thiols revealed their inter-relationship.

To establish the involvement of free radicals in causation of damage, the effect of free radical scavengers and metal chelators was studied. The activity of RP-1 was concentration dependent. It induced superoxide anions in the presence of copper ions ($CuSO_4$, 100 μM). Such auto-oxidative characteristics of flavonoids, the constituents of RP-1, have been well reported (Burkitt & Duncan 2000). Auto-oxidation of flavonoids led to formation of superoxide anions, which generated OH radicals in the presence of traces of transition metal ions and induced DNA strand breaks. RP-1 at lower concentrations ($< 400\ \mu g\ mL^{-1}$) induced single strand breaks in plasmid DNA (Figure 4) as was confirmed by the emergence of the relaxed form from the supercoiled form. However, at higher concentrations ($> 400\ \mu g\ mL^{-1}$) the appearance of linear DNA confirmed the concentration based behaviour in causation of single or double strand breaks.

The mechanism of causation of auto-oxidative damage by RP-1 was further investigated to assess the role of oxidative species present in diffused or localized sites. Glycerol and mannitol, the standard free radical scavengers, did not prevent strand breaks in plasmid DNA in the presence of RP-1. Dimethylsulfoxide, another free radical scavenger, partially inhibited strand breaks. The metal chelators (EDTA, 10 mM; deferoxamine 5 mM) strongly inhibited the strand breaks. The inability of free radical scavengers to protect plasmid DNA ruled out the involvement of free diffusible OH radicals and indicated the involvement of site-specific damage. Several agents such as flavonoids, tannins etc. have been reported to elicit site-specific DNA damage (van Acker 1998) and may explain the behaviour of RP-1. In this connection certain important known constituents of RP-1 (podophyllin, podophyllotoxin, quercetin) were investigated individually. Podophyllotoxin or quercetin, but not podophyllin, individually induced a significant amount of single strand breaks in plasmid DNA (Figure 5). Podophyllin is in fact a mixture of several compounds (Singh & Shah 1994) and some of the constituents might be interfering with its strand breaking ability. None of the three constituents could cause double strand breaks, indicating the requirement of some free metal ions which were present in RP-1 and not in the isolated constituents of RP-1. These studies indicated that to achieve desired therapeutic results the concentrations of the active ingredients need to be given proper attention.

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