

Radioprotection of Normal Tissues in Tumor-bearing Mice by Troxerutin

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Radioprotection/Troxerutin/Radiotherapy/DNA damage/Membrane damage/Lipid peroxidation/Comet assay.

The flavanoid derivative troxerutin, used clinically for treating venous disorders, protected biomembranes and cellular DNA against the deleterious effects of γ -radiation. The peroxidation of lipids (measured as thiobarbituric acid-reacting substances, or TBARS) in rat liver microsomal and mitochondrial membranes resulting from γ -irradiation up to doses of 500 Gy *in vitro* was prevented by 0.2 mM troxerutin. The administration of troxerutin (175 mg/kg body weight) to tumor-bearing mice by ip one hour prior to 4 Gy whole-body γ -irradiation significantly decreased the radiation-induced peroxidation of lipids in tissues such as liver and spleen, but there was no reduction of lipid peroxidation in tumor. The effect of troxerutin in γ -radiation-induced DNA strand breaks in different tissues of tumor-bearing mice was studied by comet assay. The administration of troxerutin to tumor-bearing animals protected cellular DNA against radiation-induced strand breaks. This was evidenced from decreases in comet tail length, tail moment, and percent of DNA in the tails in cells of normal tissues such as blood leukocytes and bone marrow, and these parameters were not altered in cells of fibrosarcoma tumor. The results revealed that troxerutin could preferentially protect normal tissues against radiation-induced damages in tumor-bearing animals.

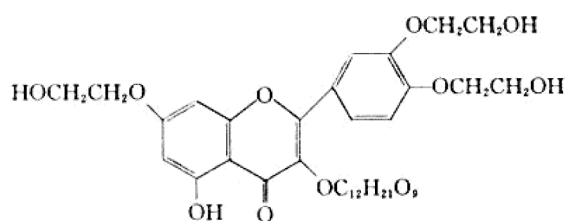
INTRODUCTION

There has been extensive research on radioprotective compounds during the past 50 years because of the relevance of these compounds in military, clinical, and industrial applications. Radiation protection might offer a tactical advantage on the battlefield in the event of nuclear warfare. Radioprotectors could reduce the cancer risk to populations exposed to radiation directly or indirectly through industrial and military applications. They are required to reduce normal tissue injury during radiotherapy of cancer. Although a variety of compounds exhibit considerable radioprotecting property in the laboratory, most of them fail in human applications because of toxicity and side effects. A search for more effective and less toxic radioprotectors has led to increasing interest in natural and synthetic pharmaceutical compounds with low toxicity profiles that can suppress the formation of free radicals.^{1–7} A wide variety of naturally occurring and synthetic drugs used for treating several illness exhibit efficient free radical scavenging properties, and these could act as radioprotectors.⁸ Recently it has been reported that histamine H₂ receptor

antagonists such as cimetidine, ranitidine, and tamolidine, besides being good inhibitors of histamine-stimulated gastric acid secretions, could offer radiation protection *in vitro* and *in vivo*.⁹

Naturally occurring antioxidant compounds such as flavanoids, polyphenols, and vitamin E offer protection against the deleterious effects of ionizing radiation because of their ability to scavenge free radicals.^{3,6,7,10,11} The flavanoid derivative troxerutin {2-[3,4-bis(2-hydroxyethoxy)phenyl]-3[(6-deoxy- α -L-manno-pyranosyl)- β (-D-glucopyranosyl)-oxy]-5-hydroxy-7-(2hydroxyethoxy)-4H-1-benzo-pyran-4-one} has been used therapeutically for treating chronic venous insufficiency (CVI),^{12–19} varicosity,²⁰ and capillary fragility.^{21–23} It has anti-erythrocytic, antithrombotic, fibrinolytic,¹³ odema-protective,²⁴ and rheological activity.^{14,17} Troxerutin scavenges oxygen-derived free radicals.^{25–28} It has been reported that during radiotherapy of head and neck cancer, the administration of a mixture of troxerutin and coumarin offered protection to salivary glands and mucosa.²⁹ The structure of troxerutin is given in Fig. 1. The present study focuses on the effect of troxerutin on gamma-radiation-induced lipid peroxidation of rat microsomal and mitochondrial membranes (*in vitro*), as well as mice liver, spleen, and tumor (*in vivo*). The effect of troxerutin on radiation-induced DNA damage in mice blood, bone marrow, and tumor cells is also studied by the use of comet assay.

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Troxerutin

Fig. 1. Structure of troxerutin.

MATERIALS AND METHODS

Materials

Troxerutin, high melting point agarose, low melting point agarose, Na₂-EDTA, Triton X-100, DMSO, Trise-base, Trypan blue, and Propidium iodide were obtained from Sigma Chemicals Inc. (St. Louis, MO, USA). All other chemicals used were of analytical grade procured locally.

Animals

Male swiss mice, 8–10 weeks old and weighing 20–25 g, were selected from an inbred group maintained under standard conditions of temperature (25 ± 2°C) and humidity. The animals were provided with food and water ad libitum. Usually, four animals were housed in each sterile polypropylene cage containing sterile paddy husk as bedding.

All the animal experiments were conducted with strict adherence to the ethical guidelines laid down by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) constituted by the Animal Welfare Division of Government of India on the use of animals in scientific research.

Tumor transplantation

For studying the influence of Troxerutin on radiation-induced lipid peroxidation and DNA strand breaks, a serially transplanted fibrosarcoma originally developed by a subcutaneous injection of 6,12-dimethylbenzo (1,2-b, 5,4-b) dithionaphthene was used as a test system.³⁰ Tumors 10–15 days old were excised and minced, and single-cell suspension was prepared in a sterile environment. About 200 µl of this murine fibrosarcoma single-cell suspension (1 × 10⁶ cells) in phosphate buffered saline was transplanted by subcutaneous injection on the dorsal side of the hind limbs of the mice. When the tumor reached a diameter of 8–10 mm, the experiment was conducted.

Drug administration

The animals were administered troxerutin of a concentration 175 mg/kg body weight in double-distilled water (DDW) intraperitoneally.

Isolation of mitochondria and microsomes

Rat liver mitochondrial and microsomal fractions were isolated by the protocol standardized in our laboratory.^{31,32} The protein concentration of the mitochondrial and microsomal fraction was determined by the Lowry method.³³

Irradiation

Rat liver mitochondrial and microsomal membranes were exposed to various doses of ⁶⁰Co-gamma rays (0–500 Gy) in a Gamma Cell 220 (AECL, Canada) at a dose rate of 6.92 Gy/min in the absence and presence of different concentrations (0.1–0.8 mM) of Troxerutin. For whole-body gamma irradiation, the animals were kept in a well-ventilated acryl box and exposed to 4 Gy in a Junior Theratron unit with a dose rate of approximately 0.5 Gy/min.

Analysis of membrane damage in vitro

The damage to mitochondrial and microsomal membranes by gamma radiation was assessed in terms of lipid peroxidation.³⁴ Mitochondrial and microsomal membranes were suspended in 250 µl of 10 mM potassium phosphate buffer pH 7.4 to have a protein equivalent of 200 to 300 µg and exposed to different doses of gamma radiation in the absence and presence of different concentrations of troxerutin. After radiation exposure, 750 µl TBA reagent [0.375% thiobarbituric acid (TBA), 0.25 M HCl, 15% trichloroacetic acid (TCA), and 6 mM EDTA] was added. The reaction mixture was incubated at 85°C for 20 min, cooled to ambient temperature, and centrifuged at 12,000 ×g for 10 min at 25°C. Thiobarbituric acid reactive substances (TBARS) in the supernatant was estimated by measuring the absorption at 535 nm by the use of a Varian DMS 200 UV-Visible spectrophotometer. Lipid peroxidation values are expressed as n moles of TBARS per mg of protein.^{31,32}

Effect of troxerutin on radiation-induced lipid peroxidation in murine tissues

The animals were divided into the following groups:

- (1) Double-distilled water (DDW) + sham irradiation
- (2) DDW + 4 Gy irradiation
- (3) Troxerutin + sham irradiation
- (4) Troxerutin + 4 Gy irradiation

One hour after the administration of DDW or Troxerutin, the animals were whole-body exposed to 0 (sham irradiation) or 4 Gy by use of a Junior Theratron unit (AECL, Canada) at a dose rate of 0.5 Gy/min in a polycarbonated cage. After two hours of irradiation, all the animals were sacrificed by cervical dislocation, and the liver, spleen, and tumor were removed and kept in ice-chilled 10 mM potassium phosphate buffer (pH 7.4). All tissues were washed in the chilled potassium phosphate buffer to remove blood. A 10% homogenate of liver, spleen, and tumor was made in the potassium phosphate buffer. This homogenate was used for assaying the lipid peroxidation as described above.

Effect of troxerutin on radiation-induced DNA damage in murine tissues

The animals were divided and irradiated as described above. They were sacrificed by cervical dislocation; blood was withdrawn from the heart with a heparinized hypodermic syringe and collected in heparinized ependorf tubes. The tumor was dissected and kept in phosphate-buffered saline (PBS), and the bone marrow cells were collected by flushing the femur bone of each animal with PBS. All the samples were stored on ice in dark.

A single cell suspension of the tumor was prepared by use of a cell dissociation sieve tissue grinder kit from Sigma, Biochemicals & Reagents, USA, with 50 mesh screen (Cat # S-0895, Sigma Chemicals, St. Louis, USA). Cell viability was tested by a trypan blue dye exclusion test with 0.02% trypan blue in PBS. To study the DNA damage in these single cell suspensions, we performed an alkaline single-cell gel electrophoresis (comet assay).

Single cell gel electrophoresis (SCGE)

The comet assay carried out was based on the method of Singh *et al.*³⁵⁾ with some modification. Fully frosted microscope slides (Gold Coin, Mumbai) were covered with 200 μ l of 1% normal melting agarose (NMA) in PBS at 45°C, immediately coverslipped and kept at 4°C for 10 min to allow the agarose to solidify. The removal of the cover glass from the agar layer was followed by the addition of a second layer of 200 μ l of 0.5% low-melting agarose (LMA) containing approximately 10^5 cells at 37°C. Cover glasses were placed immediately, and the slides were placed at 4°C. After the solidification of the LMA, the cover glasses were removed and the slides were placed in the chilled lysing solution containing 2.5 M NaCl, 100 mM Na₂-EDTA, 10 mM Tris-HCl, pH 10, and 1% DMSO, 1% Triton X100 and 1% sodium sarcosinate for 1 h at 4°C. The slides were removed from the lysing solution and placed on a horizontal electrophoresis tank filled with freshly prepared alkaline buffer (300 mM NaOH, 1 mM Na₂-EDTA, and 0.2% DMSO, pH \geq 13.0). The slides were equilibrated in the same buffer for 20 min, and the electrophoresis was carried out for 20 min at 40 V, 350 mA. After electrophoresis, the slides were washed gently with 0.4 M Tris-HCl buffer, pH 7.4, to remove the alkali. They were stained by lying on the top with 20 μ l of propidium iodide (PI, 20 μ g/ml) and visualized with a Carl Zeiss Axioskop microscope that had bright field-phase contrast and epi-fluorescence facility. The images were captured with a high-performance JVG TK 1280E color video camera. The integral frame grabber used in this system (Cvfb01p) is a PC-based card, and it accepts color composite video output of the camera. The quantification of the DNA strand breaks of the stored images was done with the imaging software SCG-Pro, developed in our research center, by which tail length, tail moment, and % DNA in the tail could be obtained directly.³⁶⁾

Statistical analysis

The results are represented as mean \pm standard error of mean (Mean \pm SEM). We performed the ANOVA by using origin 5.0. The results are significant at $p \geq 0.001$, $p \geq 0.01$, $p \geq 0.05$, and $p \geq 0.1$.

RESULTS

Protection of organelles membranes in vitro

As shown in Fig. 2, a and b, it was found that there was a dose-dependent increase in TBARS in the mitochondrial and microsomal membranes in response to an exposure of gamma radiation in vitro, and the addition of troxerutin decreased TBARS in a concentration-dependent manner. The optimum protection in both mitochondrial and microsomal membranes was observed at 0.2 mM. At this concentration of drug, the peroxidation of lipids induced by 500 Gy gamma radiation

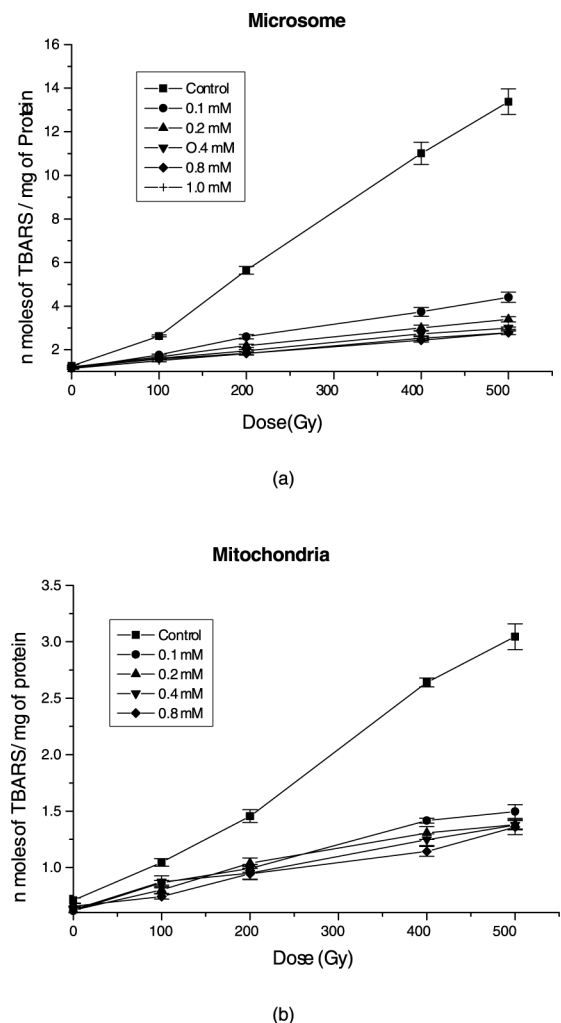


Fig. 2. Effect of Troxerutin on gamma-radiation-induced lipid peroxidation in rat liver (a) microsomal and (b) mitochondrial membranes. Each point represents the mean \pm SEM (standard error of mean).

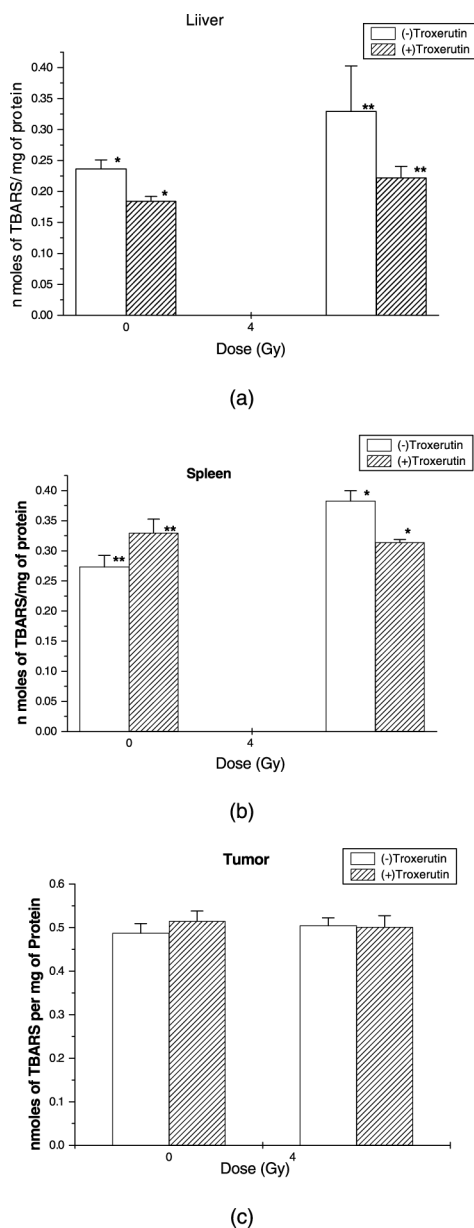


Fig. 3. Effect of troxerutin on lipid peroxidation in tissues of mice bearing fibrosarcoma tumor in terms of TBARS, (a) liver, (b) spleen, and (c) tumor following whole-body gamma radiation (4 Gy) in terms of TBARS. Results are significant at level $**p > 0.1$, $*p > 0.01$.

was decreased by 75% in microsomal membrane and 55% in mitochondrial membrane.

Protection of membranes of murine tissues in vivo

The *in vivo* study showed that there was an increase in lipid peroxidation in tissues upon an exposure to 4 Gy radiation from that of untreated control. An administration of troxerutin (175 mg/kg body weight) 1 h prior to irradiation decreased the level of lipid peroxidation. The degree of protection was

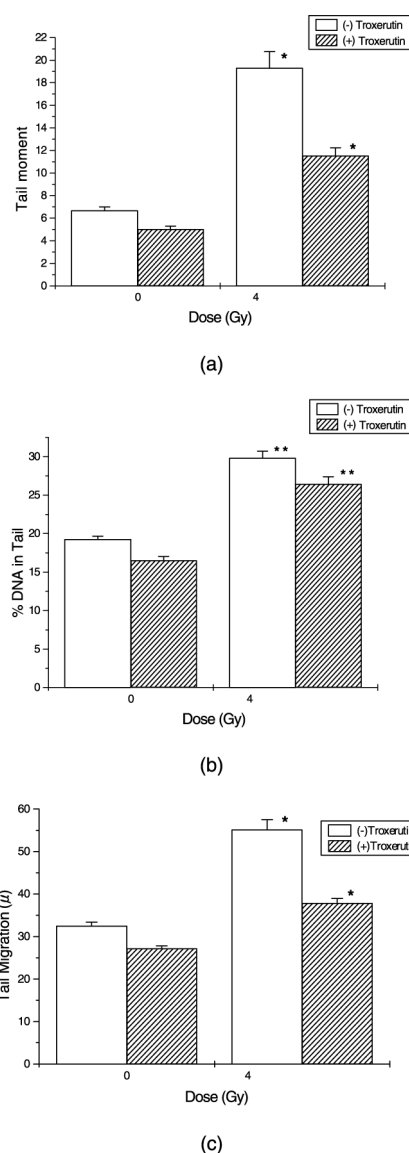


Fig. 4. Effect of troxerutin on DNA damage in murine leucocytes assayed by comet assay, (a) tail moment, (b) % DNA in tail, and (c) tail migration. Results are significant at level $*p > 0.001$ and $**p > 0.01$.

found to vary in different tissues. In regard to liver, it was found that upon whole-body exposure of 4 Gy there was increase in the level of TBARS from 0.236 ± 0.015 to 0.329 ± 0.073 , and an administration of drug to 175 mg/kg. body weight decreased it to 0.222 ± 0.018 (Fig. 3a). An administration of drug caused a slight decrease in the level of TBARS in the sham-irradiated group (0.184 ± 0.008). In spleen there was increase in the level of TBARS upon whole-body exposure (4 Gy) to 0.383 ± 0.017 , from 0.273 ± 0.019 , and an administration of the drug resulted in a reduction in level of TBARS to 0.314 ± 0.005 (Fig. 3b). There was increase in the level of TBARS in the drug-administered sham-irradiated

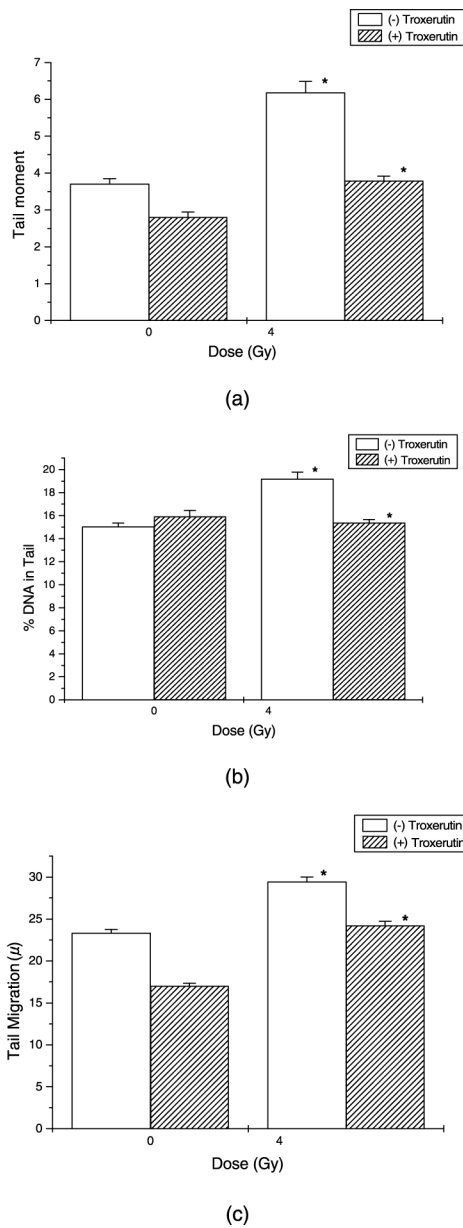


Fig. 5. Effect of troxerutin on DNA damage in murine bone marrow cells assayed by comet assay, (a) tail moment, (b) % DNA in tail, and (c) tail migration. Results are significant at level $*p > 0.001$.

group (0.33 ± 0.023) (Fig. 3b).

There was increase in the level of lipid peroxidation in tumor tissue to 0.504 ± 0.02 , from 0.487 ± 0.022 , upon whole-body exposure to gamma radiation (4 Gy). An administration of troxerutin 1 hour prior to irradiation resulted in no decrease in the level of lipid peroxidation; this is evident in Fig. 3c.

Effect of troxerutin on radiation-induced DNA damage in murine tissues

Whole-body exposure of tumor-bearing animals to gamma-

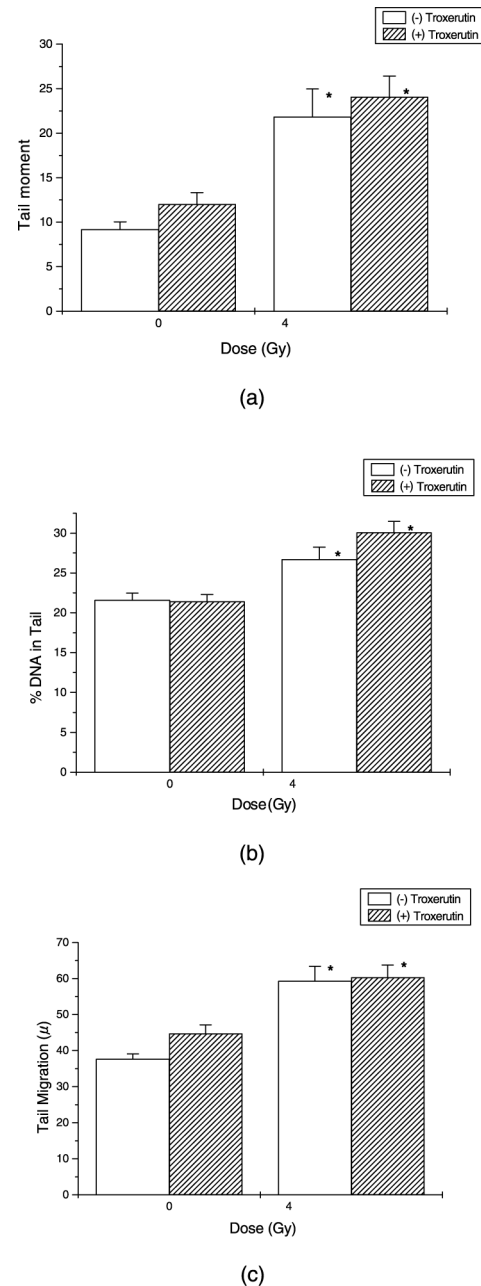


Fig. 6. Effect of troxerutin on DNA damage in fibrosarcoma tumor grown in mice, assayed by comet assay, (a) tail moment, (b) % DNA in tail, and (c) tail migration. Results are insignificant at level $*p > 0.05$.

radiation (4Gy) resulted in an increase in the comet parameters (such as % DNA in tail, tail moment, and tail migration) of cells of various tissues and tumor as a result of damage to cellular DNA (Fig. 4–6). When troxerutin was administered 1 h prior to irradiation, there was a significant decrease in comet parameters in blood leukocytes and bone marrow cells, but not in the tumor cells of irradiated animals (Fig. 6, a–c).

When animals were exposed to gamma radiation (4 Gy), in

blood cells tail moment increased to 19.28 ± 1.47 , from 6.66 ± 0.33 ; % DNA in tail increased to 29.81 ± 0.93 , from 19.24 ± 0.42 ; and % DNA in tail migration increased to 55.08 ± 2.41 from 32.43 ± 0.96 . But an administration of troxerutin 1 h prior to irradiation brought down these parameters to levels of 11.53 ± 0.72 , 26.42 ± 0.96 , and 37.78 ± 1.07 , respectively, in the irradiated group (Fig. 4, a–c). In the bone marrow cells of animals exposed to radiation (4 Gy), the tail moment and the % DNA in tail and in tail migration were increased to respective control values 6.18 ± 0.31 , 19.17 ± 0.61 , and 29.42 ± 0.59 , from 3.07 ± 0.14 , 15.02 ± 0.34 , and 23.32 ± 0.45 , respectively. The administration of troxerutin 1 h prior to irradiation brought down to levels of 3.79 ± 0.13 , 15.37 ± 0.29 , and 24.21 ± 0.54 respectively, in the irradiated group (Fig. 5, a–c). It can be seen in Fig. 6, a–c that after exposure to 4 Gy radiation, the tumor cells showed an increase in tail moment and % DNA in tail and in tail migration to 21.82 ± 3.14 , 26.67 ± 1.58 , and 59.26 ± 4.14 , from 9.16 ± 0.89 , 21.56 ± 0.91 , and 37.57 ± 1.53 , respectively. But unlike the cells of blood and bone marrow, there was no significant decrease in the comet parameters of these cells when tumor-bearing animals were exposed to radiation after troxerutin was administered.

DISCUSSION

The present study indicates that troxerutin, a cardiovascular drug, besides its antierythrocytic, antithrombotic, fibrinolytic,¹³ odema-protective,²⁴ rheological activity,^{14,17} exhibits a higher ability to protect the microsomal, mitochondrial, and normal murine tissues, liver, and spleen than the fibrosarcoma tumor cells against γ -radiation-induced lipid peroxidation. Comet assay results indicate that in tumor-bearing animals exposed to gamma-radiation, this drug offers more protection to cells of normal tissues, blood leucocytes, and bone marrow, than the tumor cells against γ -radiation-induced DNA strand breaks.

Troxerutin has undergone numerous clinical trials, animal studies, and *in vitro* studies. Even with high doses, this compound had excellent safety and tolerability profiles. In *in vitro* testing with *Salmonella typhimurium* tester strains, troxerutin was not found to be mutagenic.³⁷ In clinical trials, it has been given in doses of up to 7 g per day orally for up to 6 months with no contraindications.³⁸ There was no clinical consequence when it was given to pregnant women at a dose of 4 g per day.^{39,40} Troxerutin has been shown to be safe and effective in the treatment of chronic venous insufficiency (CVI).^{12–19} It has marked affinity for the venous wall.⁴¹ The highest uptake of the drug in the outer wall region has been reported to result from its transport through the vasorum because of the rheological properties of the drug.⁴² Troxerutin inhibits platelet adhesion to the extracellular matrix.²² This inhibition of platelet adhesion to the extracellular matrix²² yields an antierythrocyte aggregation effect and exerts a favorable action on the blood fibrinolytic system.¹³ The intramuscular admin-

istration of a combination of 150 mg of troxerutin and 1.5 mg of carbazochrome was effective in improving hemorrhoidal and postsurgical symptoms following surgery.⁴³

Membranes and DNA are the vital targets for radiation inactivation in the biological system. One of the major lesions in cellular membranes induced by ionizing radiation is lipid peroxidation. It can be initiated by radiolytic products, including hydroxyl and peroxy radicals. When microsomal and mitochondrial membranes were exposed to gamma radiation, the peroxidation of lipids increased with increasing doses of radiation. The present study revealed that troxerutin prevents radiation-induced membrane lipid peroxidation in a concentration-dependent manner. In tumor-bearing animals administered with troxerutin, there was a significant decrease in radiation-induced lipid peroxidation in liver and spleen, but no significant reduction of lipid peroxidation in tumor cells.

Ionizing radiation-induced damages to cellular DNA is of prime biological significance. The types of damage suffered by DNA as a result of ionizing radiation include strand breaks of single- and double-strand types, base damage, elimination of bases, and sugar damage.⁴⁴ Alkaline comet assay is a sensitive method by which DNA strand breaks at a single-cell level can be monitored. The comet assay results in irradiated tumor-bearing animals (Figs. 4–6) show that an administration of troxerutin 1 h prior to whole-body gamma radiation significantly decreased the comet parameters: % of DNA in tail, tail moment, and tail migration, in blood leucocytes, and in bone marrow cells, but not in tumor cells.

The present results would indicate that there could be a preferential protection of DNA and membranes in normal cells in comparison to the tumor cells when tumor-bearing animals were exposed to gamma radiation after the administration of troxerutin. Further studies on the viability of cells from normal tissues and tumor *in vivo* and *in vitro* are needed to ascertain whether the radioprotection by this drug is restricted to normal tissues. However, it may be noted that the present work is a demonstration of *in vivo* radioprotection of normal tissues, which is close to the situation in radiotherapy. The absence of discernible radioprotection in tumor cells would suggest that either troxerutin does not protect the DNA and membrane from radiation-induced lesions in tumor cells because of the difference in cellular biochemistry or because of a lack of sufficient concentration of the drug in tumor tissue to elicit radioprotection. The biodistribution of this compound in tumor and normal tissues, the hypoxic environment of the tumor, the poor vasculature in the tumor, and the variations in the physiological and biochemical status of the cells of the tumor compared to normal cells at the time of irradiation, among other possibilities, could also be contributory factors for the observed differences in radioprotection in the normal and tumor cells. Further studies, however, are needed to determine the actual mechanism underlying the preferential radioprotection in normal tissues by this drug. Nevertheless, the present study does indicate the potential application of

troxerutin as an adjuvant in radiotherapy, since the administration of this compound an hour prior to radiation exposure results in a positive benefit to normal tissue protection.

Radiation therapy is one of the most common modalities of treatment for human cancer. To obtain better tumor control with higher doses of radiation, the normal tissues should be protected against radiation injury. Thus radioprotecting compounds are of importance in clinical radiation therapy.¹⁾ Although a large variety of compounds have shown promise as radioprotectors in laboratory studies, most of them failed even before reaching the preclinical stage because of toxicity and side effects. For a clinical application of any compound as a radioprotector, it would require absolute certainty about the protection factors for tumor and normal tissues to avoid unacceptable clinical risk. The only compound currently used as an adjuvant in radiotherapy for protecting normal tissues is amifostine, or WR2721, though there have been reports about contraindications in some cases.⁴⁵⁾ Troxerutin has been used in clinical practice for more than 3 decades to treat CVI,¹²⁻¹⁹⁾ capillary fragility,²¹⁻²³⁾ and varicosity.²⁰⁾ This compound has been found to be safe and well tolerated at high concentrations in human subjects. The present study reveals that troxerutin administration protects cells of normal tissues from deleterious effects of gamma radiation while offering no protection to tumor cells in tumor-bearing animals exposed to whole-body radiation. Thus the present work suggests that troxerutin could be used as an ideal adjuvant in radiotherapy to protect normal tissues.

ACKNOWLEDGEMENTS

The authors thank Mr. P. M. Gonsalves for his help in the animal experiments.

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Received on September 18, 2003
 1st Revision on October 31, 2003
 2nd Revision on December 9, 2003
 3rd Revision on January 19, 2004
 Accepted on January 20, 2004