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Protection of cellular DNA from γ-radiation-induced damages and enhancement in DNA repair by troxerutin

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Abstract

The effect of troxerutin on γ-radiation-induced DNA strand breaks in different tissues of mice *in vivo* and formations of the micronuclei were studied in human peripheral blood lymphocytes *ex vivo* and mice blood reticulocytes *in vivo.* Treatments with 1 mM troxerutin significantly inhibited the micronuclei induction in the human lymphocytes. Troxerutin protected the human peripheral blood leucocytes from radiation-induced DNA strand breaks in a concentration dependent manner under *ex vivo* condition of irradiation (2 Gy). Intraperitoneal administration of troxerutin (175 mg/kg body weight) to mice before and after whole body radiation exposure inhibited micronuclei formation in blood reticulocytes significantly. The administration of different doses (75, 125 and 175 mg/kg body weight) of troxerutin 1 h prior to 4 Gy γ-radiation exposure showed dose-dependent decrease in the yield of DNA strand breaks in murine blood leucocytes and bone marrow cells. The dose-dependent protection was more pronounced in bone marrow cells than in blood leucocytes. Administration of 175 mg/kg body weight of the drug (i.p.) 1 h prior or immediately after whole body irradiation of mice showed that the decrease in strand breaks depended on the post-irradiation interval at which the analysis was done. The observed time-dependent decrease in the DNA strand breaks could be attributed to enhanced DNA repair in troxerutin administered animals. Thus in addition to anti-erythrocytic, anti-thrombic, fibrinolytic and oedema-protective rheological activity, troxerutin offers protection against γ-radiation-induced micronuclei formation and DNA strand breaks and enhances repair of radiation-induced DNA strand breaks. (Mol Cell Biochem **280:** 57–68, 2005)

Key words: comet assay, DNA damage, micronuclei, radioprotection, radiotherapy, troxerutin

Introduction

One of the major challenges in radiation biology is to protect man and animals from deleterious effects of ionizing radiation [1–4]. Several plant compounds particularly flavonoids possessing high antioxidant activity are reported to have radio-protecting property [1, 5]. The flavonoids, apigenin, orientin, vicenin, luteolin, etc. were shown to inhibit radiation-induced chromosomal aberrations and formation of micronuclei [5–8]. Damage to haemopoetic system is considered to be the major hazard in mammals exposed to ionizing radiation in the low dose range. In this report we focus our studies on the effect of troxerutin, which is a flavonoid derivative, used for the treatment of certain disorders of the vascular system, on formation of micronuclei in human peripheral blood lymphocytes (*ex vivo*) and

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mice blood reticulocytes (*in vivo*) exposed to γ-radiation as well as on radiation-induced DNA damage in bone marrow and blood leucocytes of mice exposed to 4 Gy whole body γ-irradiation.

Troxerutin (Scheme 1), a derivative of the natural flavonoid rutin extracted from *Sophora japonica* (Japanese pogoda tree) has been commonly used in the treatment of Chronic Venous Insufficiency (CVI) disease [9–16]. It improves capillary function, reduces capillary fragility [17–19] and abnormal leakage, and it has anti-erythrocytic, anti-thrombotic, fibrinolytic [10], odema-protective [20] and rheological activity [11, 14]. Its effectiveness and safety has been evaluated in both elderly patients [21] as well as pregnant women [22], with excellent results. It scavenges oxygen derived free radicals [23–26]. It has been reported that during radiotherapy of head and neck cancer, administration of a mixture of troxerutin and coumarin offered protection to salivary glands and mucosa [27]. Earlier studies have shown that troxerutin inhibited lipid peroxidation in membrane of sub-cellular organelles as well as normal tissues of tumour-bearing mice exposed to γ-radiation. Further, it was found that administration of troxerutin resulted in differential protection of DNA in blood leucocytes and bone marrow cells and not in cells of tumour in whole body irradiated tumour-bearing mice [28].

Materials and methods

Materials

Troxerutin was obtained from Aldrich Chemical Company Inc., Milwaukee, WI, USA. High melting point agarose, low melting point agarose, Na_2-EDTA, Triton X-100, DMSO, Tris-base and Propidium iodide were obtained from Sigma Chemical Co Inc. (St. Louis, MO, USA). All other chemicals used were of analytical grade procured locally.

Animals

Male swiss mice, 8–10 weeks old weighing 20–25 g, were selected from inbred group maintained under standard conditions of temperature (25 $\pm$ 2 °C) and humidity. 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, Government of India on the use of animals in scientific research.

γ-Irradiation

A ^{60}Co-Junior Theratron Teletherapy unit (AECL, Otawa, Canada) was used for all irradiation work. The dose rate was 0.39 Gy/min at 38 cm distance.

Drug administration

Animals were administered different doses of troxerutin (75, 125 and 175 mg/kg body weight) in double-distilled water (DDW) intraperitoneally.

Micronuclei assay in human blood lymphocytes

Experiments were performed with blood samples from three healthy non-smoking volunteers aged between 28 and 55 years. Peripheral blood was collected in sterile vacutainer containing heparin as an anti-coagulant. The stock solution of troxerutin was prepared in phosphate buffered saline (PBS, pH 7.4) and filter sterilized using millipore syringe filter (0.22 μm). Troxerutin (150 μl of 10 mM stock solution) was added to the blood (1.35 ml) making the final concentration 1 mM. Blood samples were incubated for 25–30 min at 37 °C in CO_2 incubator and subsequently exposed to 2 Gy γ-radiation. After irradiation the lymphocytes were washed with culture medium and the cells were cultured with mitogen stimulation to determine the extent of radiation-induced genetic damage. Cultures were set up using 0.5 ml of whole blood and 4.5 ml of RPMI 1640 medium supplemented with 15% fetal calf serum, 1% reconstituted phytohaemagglutinin (PHA), 125 mM L-glutamine for 72 h. Cytochalasin B (6 μg/ml) was added to the cultures at 44 h, after the initiation of the cultures and cultures were terminated at 72 h with mild hypotonic treatment (chilled KCl 75 mM) and fixed in 4:1 methanol–acetic acid. After three washes the cells were gently dropped on a wet slide and stained with 2% Giemsa in Sorenson's buffer pH 6.8 for 10–12 min. All the slides were coded and evaluated at 1000$\times$ magnification for the frequency of micronuclei in cytokinesis blocked binucleated (BN) cells with well-preserved cytoplasm. A total of 1000 BN cells were analysed from each experimental sample as described earlier [29].

Micronuclei assay in mice blood reticulocytes in vivo

Preparation of acridine orange (AO)-coated glass slides
AO-coated slides were made according to Hayashi *et al.* [30]. Acridine orange was dissolved in distilled water at a concentration of 1 mg/ml. Ten microlitres of this solution was placed on a pre-heated (about 70 °C) cleaned glass slide. The solution was spread by moving a glass rod back and forth over

it and dried by air. The AO-coated glass slides were stored at room temperature under dry conditions.

Collection of peripheral blood cells from mice for micronuclei assay

Peripheral blood was collected by tail vein puncture (at 24 and 48 h) and 5 μl aliquots of the blood were kept on AO-coated slides and covered with cover slips. The reticulocytes (RETs) and micronucleated reticulocytes (MN-RETs) were monitored using a Carl Zeiss Fluorescent microscope with Axioskop with blue filter. A suitable region of the slides was selected under low magnification and approximately 1000 reticulocytes were scored for micronuclei.

Effect of troxerutin on radiation-induced DNA damage in human blood leucocytes

For studying the effect of troxerutin on human blood leucocytes, blood was collected from three healthy and non-smoker volunteers having the mean age 25 ± 2 years by finger prick method and stored in heparinized eppendorf tubes at ice temperature. Troxerutin was added to 100 μl blood, 5–10 min before irradiation, and the blood was exposed to 2 Gy γ-radiation. Radiation-induced damage to DNA in the blood leucocytes was measured as strand breaks using single cell gel electrophoresis (comet assay) based on the method of Singh [31] with some modification [28, 32]. In brief, frosted microscope slides (Gold Coin, Mumbai, India) were covered with 200 μl of 1% normal melting agarose (NMA) in PBS at 45 °C, immediately coversliped and kept at 4 °C for 10 min to allow the agarose to solidify. Removal of cover slip from the agar layer was followed by addition of a second layer of 200 μl of 0.5% low melting agarose (LMA) containing approximately 10^5 cells at 37 °C. Cover slips were placed immediately and the slides were placed at 4 °C. After solidification of the LMA, the cover slips were removed and slides were placed in the chilled lysing solution containing 2.5 M NaCl, 100 mM Na_2-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 Na_2-EDTA and 0.2% DMSO, pH $\geq$13.0). The slides were equilibrated in the same buffer for 20 min and electrophoresis was carried out at 25 V, 180 mA for 20 min. After electrophoresis the slides were washed gently with 0.4 M Tris–HCl buffer, pH 7.4, to remove the alkali. The slides were stained with 50 μl of propidium iodide (PI, 20 μg/ml) and visualized using a Carl Zeiss Fluorescent microscope (Axioskop) with bright field phase-contrast and epi-fluorescence facility. The images (50–60 cells/slide) were captured with high-performance JVG TK 1280E colour video camera. The integral frame grabber used in this system (Cvfbo1p) is a PC-based card and it accepts colour composite video output of the camera. The quantification of the DNA strand breaks of the stored images was done using the CASP software by which % DNA in tail, tail length, tail moment and Olive tail moment could be obtained directly [33].

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

The animals were divided into the following groups:

1. DDW + sham-irradiation
2. DDW + 4 Gy irradiation
3. Troxerutin (175 mg/kg body weight) + sham-irradiation
4. Troxerutin (175 mg/kg body weight) + 4 Gy irradiation

One hour after the administration of DDW or troxerutin (175 mg/kg body weight) animals were whole body exposed to 0 Gy (sham-irradiation) or 4 Gy in polycarbonated cage. Animals were sacrificed by cervical dislocation 2 h after post-irradiation; blood was withdrawn from heart using a heparinized hypodermic syringe and collected in heparinized eppendorf tubes, whereas bone marrow cells were collected by flushing the femur bones of each animal with PBS (pH 7.4). All the samples were stored on ice in dark until alkaline single cell gel electrophoresis was carried out.

Effect of different concentrations of troxerutin on murine tissues upon γ-radiation exposure

The animals were divided into the following groups:

(1) DDW + sham-irradiation
(2) DDW + 4 Gy irradiation
(3) Troxerutin (75 mg/kg body weight) + 4 Gy irradiation
(4) Troxerutin (125 mg/kg body weight) + 4 Gy irradiation
(5) Troxerutin (175 mg/kg body weight) + 4 Gy irradiation

One hour after the administration of DDW or different doses of troxerutin (75, 125 and 175 mg/kg body weight) animals were whole body exposed to 0 Gy (sham-irradiation) or 4 Gy in polycarbonated cage. Blood was collected at 0, 30 and 60 min from tail vein of mice. Animals were sacrificed 2 h after radiation exposure in same way as mentioned earlier and blood and bone marrow cells were taken out and alkaline single cell gel electrophoresis was carried out.

Table 1. Effect of troxerutin on γ-radiation induced inhibition of micronuclei formation in human blood lymphocytes (volunteers' ages range 28–55 yrs)

Sex	Age (Yrs)	Treatments	Micronuclei/1000 BN	% Inhibition	Average % inhibition (mean ± S.E.M.)
Male	28	Control	10	43.88	41.26 ± 3.46 $p < 0.01$
		1 mM Troxerutin	12		
		2 Gy	360		
		2 Gy + 1 mM Troxerutin	202		
Male	55	Control	8	34.40	
		1 mM Troxerutin	12		
		2 Gy	366		
		2 Gy + 1 mM Troxerutin	240		
Female	48	Control	12	45.50	
		1 mM Troxerutin	10		
		2 Gy	356		
		2 Gy + 1 mM Troxerutin	194		

Effect of troxerutin on DNA repair of mice blood leucocytes upon γ-radiation exposure

The mice were exposed to 4 Gy γ-radiation and blood was collected from tail vein just after irradiation (for 0 min) and animals were divided in two groups, one group was treated with DDW and other group was administered 175 mg/kg body weight of troxerutin immediately after radiation exposure. Blood was collected at 15, 30 and 90 and 120 min post-irradiation from tail vein. The blood samples were subjected to alkaline comet assay as discussed above to monitor the DNA strand breaks.

Statistical analysis

The data was imported to excel work sheets, and graphs were made using Origin version 5.0. Kruskal-wallis non-parametric statistics with Dunn's multiple comparison test was used to study the significant level. Data was significant at p-value less than 0.05. Each point represented as mean ± S.E.M. (standard error of mean).

Results

Effect of troxerutin on γ-radiation-induced inhibition of micronuclei formation in human blood lymphocytes

Exposure of human peripheral blood leucocytes to 2 Gy γ-radiation resulted in considerable increase in the induction of micronuclei, the number of micronuclei induction increased from 10 ± 1.15 to 360.7 ± 2.9 (Table 1). Lymphocytes treated with 1 mM troxerutin and exposed to 2 Gy γ-radiation exhibited a significant decrease in the induction of micronuclei. The percentage inhibition of micronuclei by 1 mM troxerutin upon 2 Gy γ-radiation exposure was 41.26.

Effect of troxerutin on γ-radiation-induced inhibition of micronuclei formation in mice peripheral blood reticulocytes

It can be seen in Table 2 that when animals were exposed to 4 Gy γ-radiation there was an increase in the micronuclei in blood reticulocytes at 24 and 48 h but the

Table 2. Effect of troxerutin (175 mg/kg body weight administered 1 h prior (A) and immediately after (B) 4 Gy whole body γ-radiation exposure) on γ-radiation-induced inhibition of micronuclei formation in mice peripheral blood reticulocytes

Groups	Mean ± S.E.M. of MN-RETs/1000 RETs	
	24 h	48 h
(A)		
Control	2.5 ± 0.44	1.7 ± 0.10
Trox control	3 ± 0.41	3.15 ± 0.11
4 Gy + DW	$27.30 \pm 1.15^{**}$	$38.67 \pm 0.67^{**}$
4 Gy+Trox 175 mg/kg body weight	$13.62 \pm 0.32^{**}$	$22.90 \pm 2.6^{**}$
(B)		
Control	2.5 ± 0.44	1.7 ± 0.10
Trox 175 mg/kg body weight	3 ± 0.41	3.15 ± 0.11
4 Gy + DW	$11.28 \pm 0.20^{**}$	$34.72 \pm 2.43^{**}$
4 Gy+Trox 175 mg/kg body weight	$7.41 \pm 0.33^{**}$	$23.82 \pm 2.32^{**}$

Note. $N = 4$, $^{**}P < 0.001$.

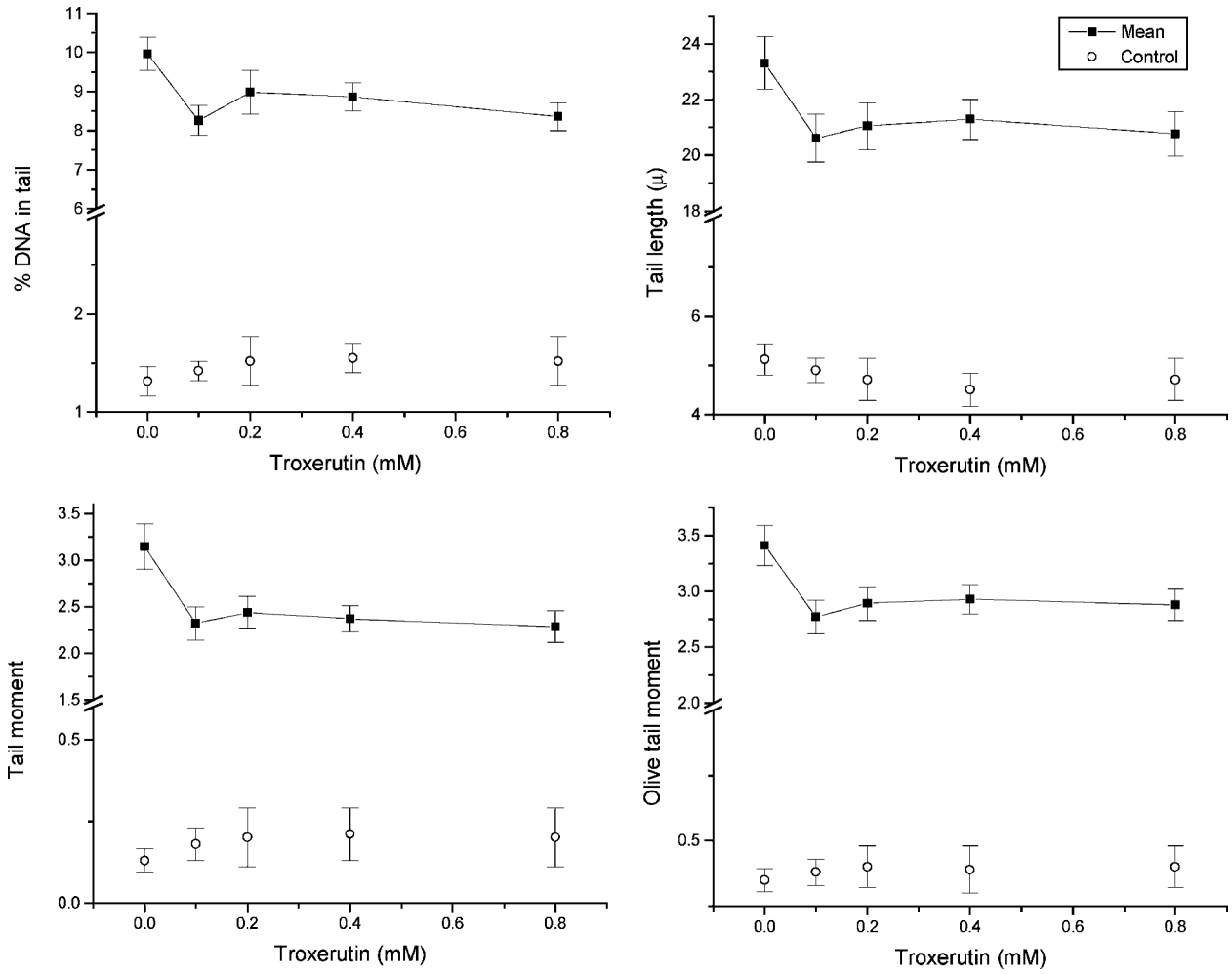

Fig. 1. Effect of various concentrations of troxerutin on γ-radiation (2 Gy) induced DNA damage in human blood leucocytes, estimated by comet assay. Mean of the % DNA in tail, tail length, tail moment, and Olive tail moment of single cells of human blood leucocytes subjected to alkaline single cell gel electrophoresis in presence of various concentrations of troxerutin are presented with ±S.E.M.

administration of troxerutin (175 mg/kg body weight) significantly ($p < 0.001$) reduced the number of micronuclei formation.

Effect of different concentrations of troxerutin on γ-radiation-induced DNA damage in human peripheral blood leucocytes

Exposure of human peripheral blood leucocytes to γ-radiation causes strand breaks and other damages in the cellular DNA. Comet assay monitors strand breaks in the cellular DNA [31]. From Fig. 1 it can be seen that when human blood leucocytes were exposed to 2 Gy γ-radiation, there was an increase in comet parameters, such as % DNA in tail, tail length, tail moment and Olive tail moment, and the presence of 0.1 mM troxerutin during irradiation significantly reduced these parameters. The results revealed that the presence of 0.1 mM troxerutin decreased the tail length and the Olive tail moment by 11.5 and 18.66% respectively in human blood leucocytes exposed to 2 Gy γ-radiation. Increasing the concentration of troxerutin from 0.1 mM to 0.8 mM did not show further reduction in the comet parameters. Incubation of unirradiated cells with troxerutin at concentrations up to 0.8 mM did not result in any alteration of the comet parameters from that of the control unirradiated cells.

Effect of troxerutin on γ-radiation-induced DNA damage in murine blood leucocytes and bone marrow cells

Damage to cellular DNA in blood leucocytes and bone marrow cells upon whole body exposure of mice to γ-radiation was studied after 2 h post-irradiation. Whole body exposure

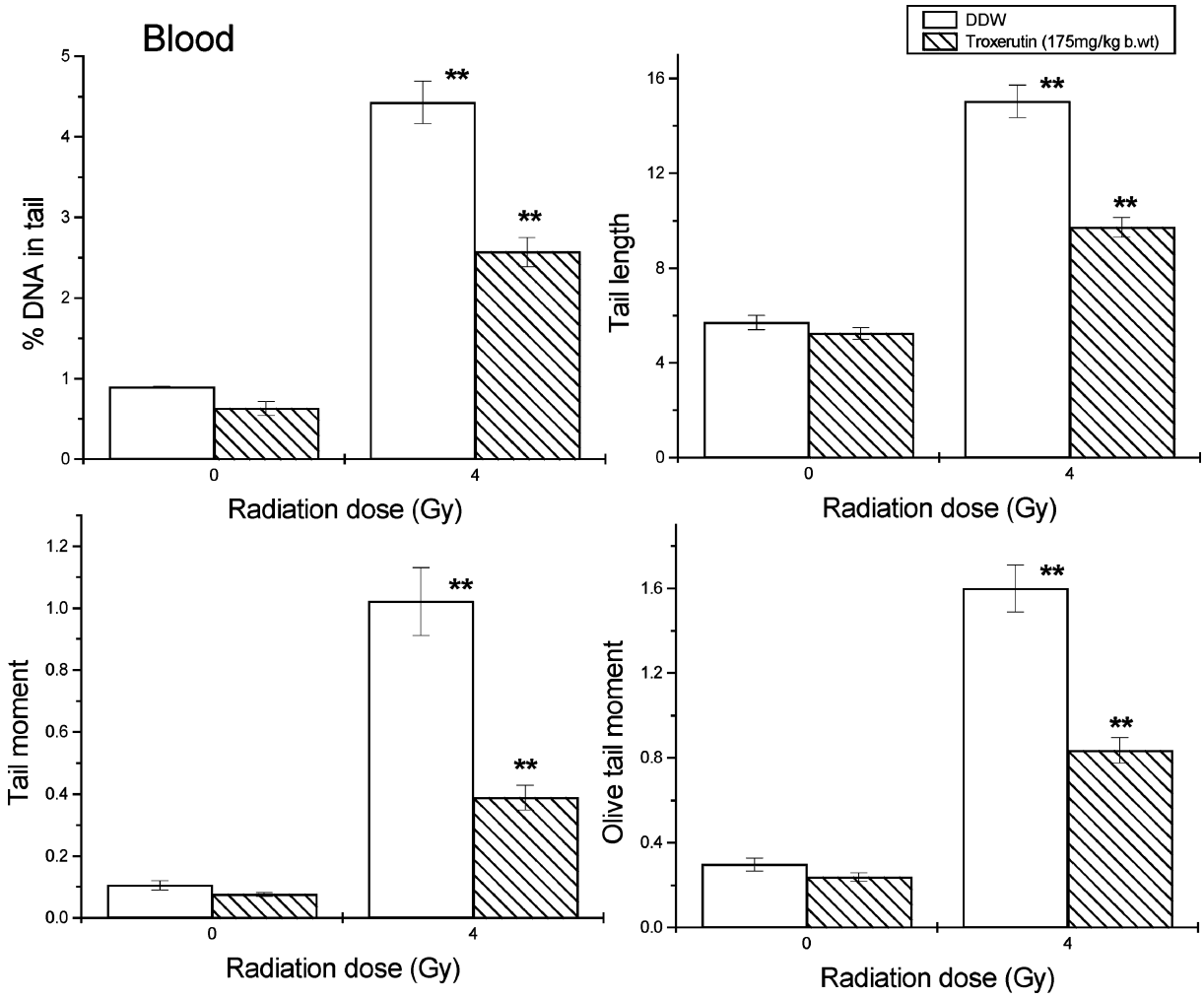

Fig. 2. Effect of troxerutin on DNA damage in murine blood leucocytes assayed by comet assay. Mean comet parameters: % DNA in tail, tail length, tail moment and Olive tail moment of single cells of blood leucocytes subjected to alkaline single cell gel electrophoresis after administration of 175 mg/kg body weight troxerutin at 2 h post-radiation are presented with ±S.E.M. ($N = 4$, $^{**}p < 0.001$).

of mice to 4 Gy γ-radiation resulted in increase in comet parameters as can be evidenced in Figs. 2 and 3. Administration of troxerutin (175 mg/kg body weight) i.p. 1 h prior to γ-radiation exposure resulted in significant ($p < 0.001$) reduction of the comet parameters (Figs. 2 and 3).

When animals were exposed to γ-radiation (4 Gy), % DNA in tail was increased from 0.894 ± 0.01 to 4.428 ± 0.26, tail length was increased from 5.71 ± 0.03 to 15.044 ± 0.69, tail moment was increased from 0.105 ± 0.016 to 1.021 ± 0.11 and Olive tail moment was increased from 0.298 ± 0.031 to 1.599 ± 0.11 in blood cells (Fig. 2). Intraperitoneal administration of troxerutin 1 h prior to irradiation brought down these parameters to a level of 2.569 ± 0.18, 9.724 ± 0.42, 0.388 ± 0.04 and 0.835 ± 0.06, respectively in the irradiated group (Fig. 2). In bone marrow cells of animals exposed to the radiation, % DNA in tail, tail length, tail moment and Olive tail moment were increased from the control values of 0.812 ± 0.1, 5.56 ± 0.34, $0.123 \pm .02$ and 0.273 ± 0.04 to 3.413 ± 0.29, 15.2 ± 1.04, 1.204 ± 0.17 and 1.384 ± 0.13, respectively (Fig. 3). The administration of troxerutin 1 h prior to the radiation exposure brought down these comet parameters in bone marrow cells to 0.71 ± 0.09, 5.4 ± 0.26, 0.089 ± 0.02 and 0.242 ± 0.03 (Fig. 3).

There was a progressive reduction of the comet parameters due to increasing doses of troxerutin from 75 mg/kg body weight 1 h prior to whole body γ-irradiation (Fig. 4). Administration of troxerutin at a dose of 175 mg/kg body weight did not alter comet parameters in unirradiated animals.

The concentration-dependent radioprotection of cellular DNA in blood leucocytes and bone marrow cells of whole body irradiated animals were plotted for best fit statistical model using program Micrococcal Origin 5.0. It can be seen

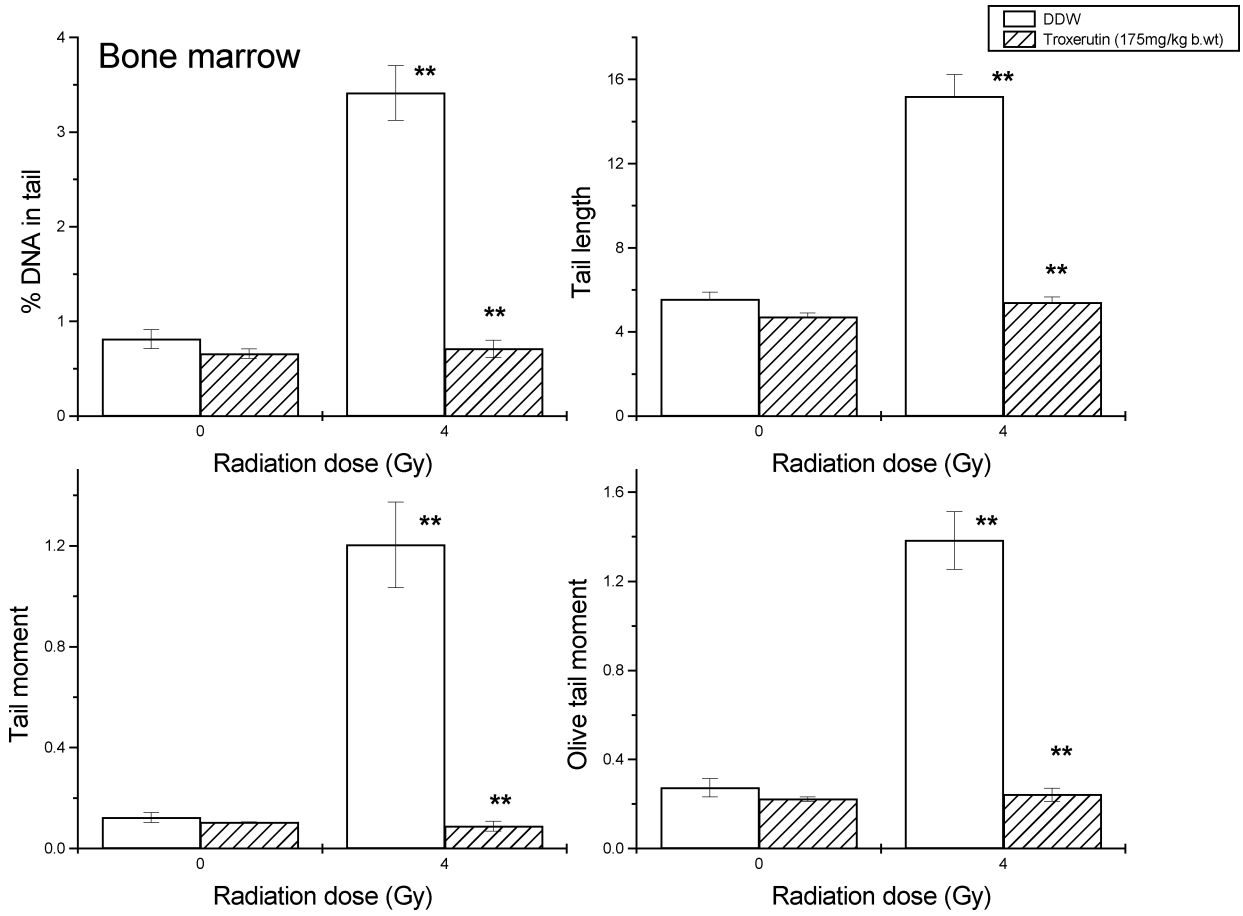

Fig. 3. Effect of troxerutin on DNA damage in murine bone marrow cells assayed by comet assay. Mean comet parameters: % DNA in tail, tail length, tail moment and Olive tail moment of single cells of bone marrow subjected to alkaline single cell gel electrophoresis after administration of 175 mg/kg body weight troxerutin at 2 h post-radiation are presented with ±S.E.M. ($N = 4$, $^{**}p < 0.001$).

in Fig. 5 that the Olive tail moment in blood leucocytes decreased linearly with increasing doses of troxerutin administration. This saturation value of radiation protection has not reached in blood leucocytes even at the administering of a dose of 175 mg/kg body weight troxerutin [$R = -0.97603$, $Y = 1.54 + (-0.00433X)$, $p < 0.02$], whereas in bone marrow it decreased exponentially with increasing dose of troxerutin as discernible in Fig. 5.

As these observations were made at 2 h post-irradiation, it was not certain whether the observed reduction in the comet parameters could be due to protection against initial radiation damage in DNA as well as due to DNA repair. It is evident from the Fig. 6 that troxerutin administration reduced the radiation-induced damage to cellular DNA as at 0 min the comet parameters were lower than that of the irradiated untreated group. However it could be argued that the pronounced decrease of the comet parameters observed in the irradiated troxerutin-treated group could be attributed the initial low level of DNA damage rather than enhancement in DNA repair. To resolve this and to examine the specific effect of troxerutin on DNA repair, studies on analysis of DNA damage were undertaken following post-irradiation administration of troxerutin.

To examine the effect of troxerutin administration on repair of cellular DNA, the comet parameters of blood leucocytes of whole body irradiated mice were examined at different time intervals from 0 to 120 min. It can be seen in Fig. 6 that there was a decrease in comet parameters with increase in time of post-irradiation indicative of time-dependent DNA repair. In the troxerutin-treated animals the decrease of the comet parameters was found to be more pronounced than that of the untreated animals.

Effect of troxerutin administration on repair of radiation-induced damage to cellular DNA

The effect of troxerutin on DNA repair was ascertained by examining the comet parameters of the peripheral blood

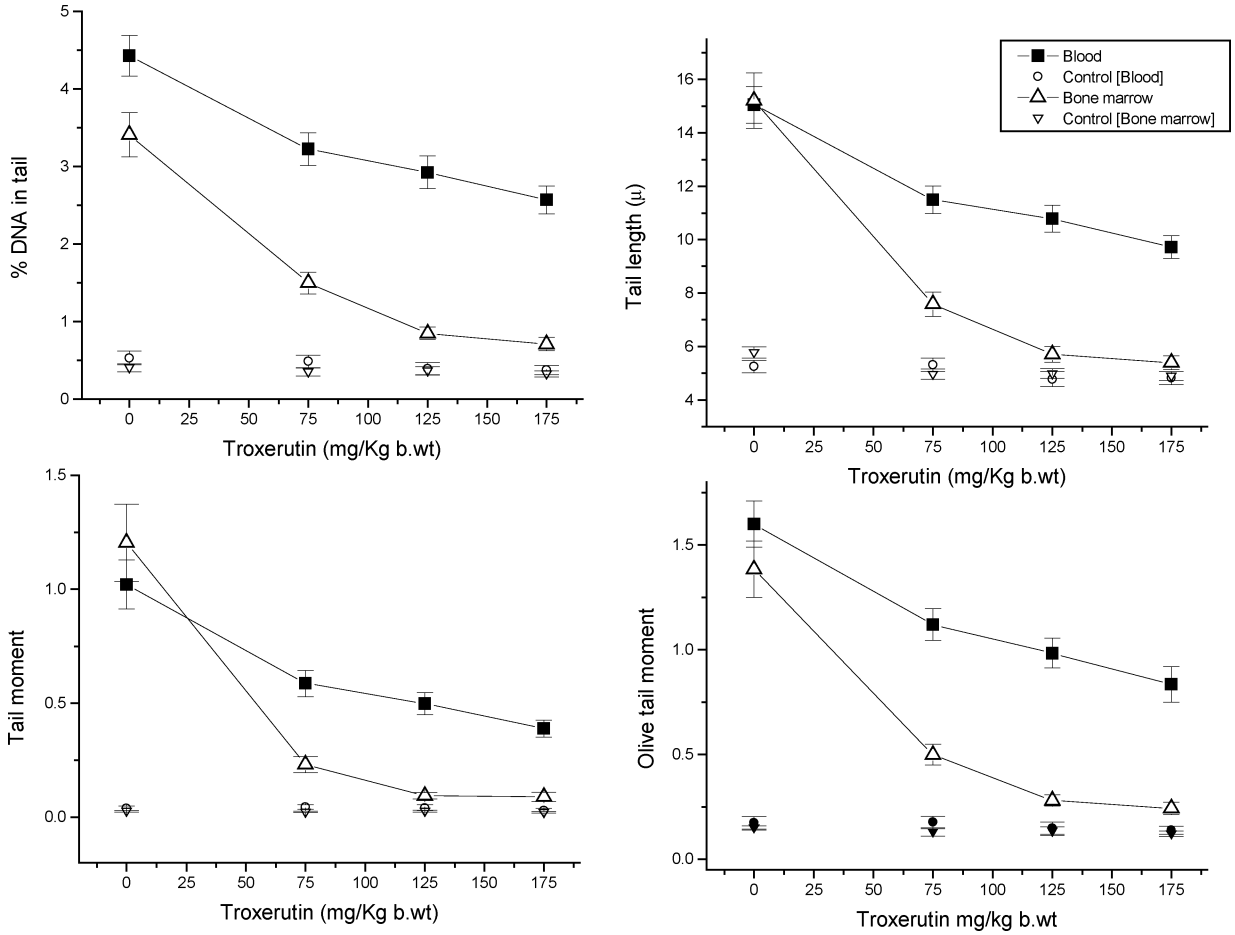

Fig. 4. Effect of troxerutin on DNA damage in murine blood leucocytes and bone marrow cells assayed by comet assay. Mean comet parameters: % DNA in tail, tail length, tail moment and Olive tail moment of single cells of blood leucocytes and bone marrow cells subjected to alkaline single cell gel electrophoresis after administration of various doses of troxerutin at 2 h post-radiation are presented with ± S.E.M. ($N = 4$, $p < 0.0001$).

leucocytes from whole body irradiated mice at different intervals following post-irradiation after administration of troxerutin (175 mg/kg body weight) and the results are presented in Fig. 7. In the troxerutin-treated animals the decrease of the comet parameters was more than that of the untreated animals. From Fig. 7 it can be seen that during the initial 15 min, repair of most of the DNA damages was completed. The repair of DNA takes place at a faster rate in troxerutin-treated animals than in untreated ones. Both in irradiated control group and troxerutin-treated group the comet parameters exhibited a peak at 30 min post-irradiation, which could be due to the commencement of excision repair process [34]. This repair system involves removal of the DNA lesions such as radiation-induced base modification through a process of incision and excision at the site of damage in DNA strand leading to generation of strands breaks [34]. Some of the breaks resulting from excision repair process are subsequently repair. The cells with un-repaired lesions may go for apoptotic pathway. The increase in comet parameters in later time interval (120 min) may be a reflection of the apoptotic process in cells having un-repaired lesions. Thus the results would indicate that apart from offering protection to cellular DNA against radiation, troxerutin also enhanced the cellular DNA repair process.

Discussion

The present study reveals that troxerutin, a cardiovascular drug, in addition to its anti-erythrocytic, anti-thrombotic, fibrinolytic [10], odema-protective [20], rheological activity [11, 14], has radio-protective and DNA repair enhancement property.

Ionizing radiation-induced damages to cellular DNA is of prime biological significance. The types of damage suffered by DNA due to ionizing radiation include strand breaks of

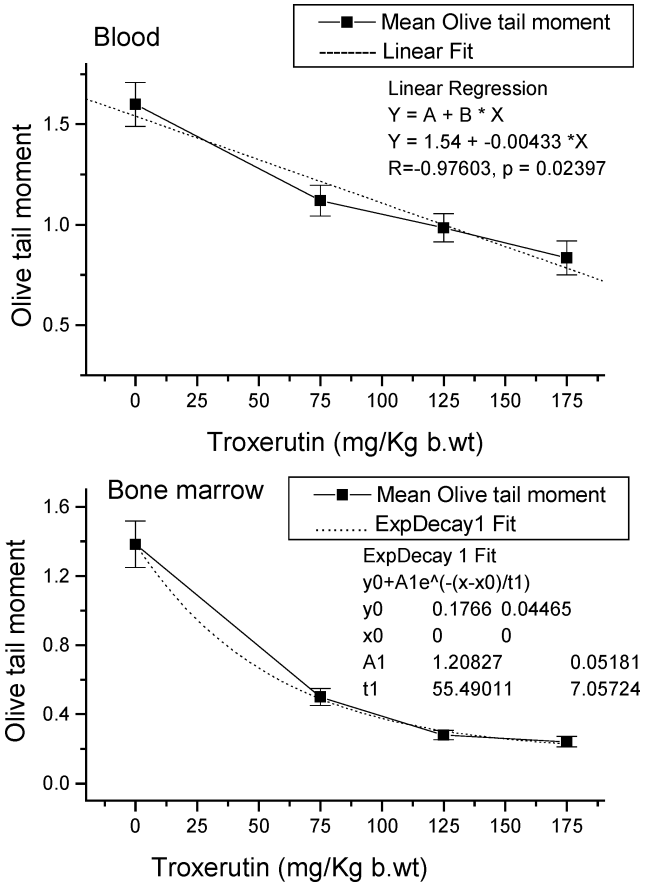

Fig. 5. Statistical analysis for best fit of Olive tail moment of single cells of murine tissues – blood and bone marrow subjected to single cell gel electrophoresis.

single and double-strand types, base damage, elimination of bases and sugar damage [35]. DNA strand breaks of the double strand type lead to the formation of micronuclei. Cytokinesis block micronucleus assay is an excellent method to monitor the micronuclei formation in the blood lymphocytes [29]. Troxerutin inhibits radiation-induced micronuclei formation in *ex vivo* exposed human peripheral blood lymphocytes. Our result showed that presence of 1 mM troxerutin during radiation exposure (2 Gy) inhibited micronuclei by 41.26%. The *in vivo* study of micronuclei inhibition in the mice blood reticulocytes also showed that troxerutin significantly ($p < 0.001$) inhibited the formation of micronuclei when troxerutin was administered prior and after radiation exposure (4 Gy).

Alkaline comet assay is a sensitive technique to monitor DNA strand breaks and alkali labile DNA lesions, and is widely used to study genotoxicity [36] and lesions in cellular DNA such as single and double strand breaks, DNA repair and apoptosis induced by environmental toxic agents [37–41]. The present study demonstrates that presence of 0.1 mM of troxerutin significantly reduced the DNA strands breaks in human peripheral blood leucocytes exposed to γ-radiation *ex vivo*. The *in vivo* results from murine system indicate that administration of troxerutin 1 h prior to whole body γ-radiation (4 Gy) protects cellular DNA in peripheral blood leucocytes and bone marrow cells from radiation-induced damage in a dose-dependent manner. In peripheral blood leucocytes, the damage to DNA, as evidenced by Olive tail moment, linearly decreased with increasing doses of troxerutin administration even up to 175 mg/kg body weight, while in bone marrow cells the decrease in Olive tail moment showed an exponential relationship. This would suggest that administration of troxerutin up to 175 mg/kg body weight leads to saturation level of DNA protection in bone marrow cells while it has not reached such levels of saturation in blood cells. This could be an inherent property of peripheral blood leucocytes as these cells are often subjected to oxidative and metabolic stresses which may also introduce some lesions in cellular DNA.

No literature is available about enhancement of DNA repair by radio-protectors, except tocopherol monoglucoside (TMG), in *Saccharomyces cerivisae* [42]. The present results indicate that DNA repair is enhanced in peripheral blood leucocytes of whole body irradiated animals administered with troxerutin (175 mg/kg body weight) as evidenced from the comet parameters. The repair can be due to troxerutin or its metabolic derivatives in the blood. At present we do not have data with respect to the metabolic derivatives of troxerutin.

Troxerutin has undergone numerous clinical trials in human subjects. Even with high doses, troxerutin had excellent safety and tolerability profiles [17, 21, 22]. In *in vitro* testing with *Salmonella typhimurium* tester strains, troxerutin did not show any mutagenicity [43]. In our studies on micronuclei induction also corroborate that troxerutin does not have genotoxicity as the drug did not significantly increase the micronuclei in controls. Radiation-induced micronuclei induction was inhibited by troxerutin under *in vitro* (human peripheral lymphocytes) and under *in vivo* condition (mice blood reticulocytes) indicating ability of troxerutin to protect from radiation-induced genomic instability. In clinical trials, troxerutin has been given in doses up to 7 g per day orally for up to 6 months with no contraindications [17]. There was no clinical consequence when troxerutin was given to pregnant women at a dose of 4 g per day [21, 22]. Troxerutin have been shown to be safe and effective in treatment of chronic venous insufficiency (CVI) disease [9–16]. It has marked affinity for the venous wall [44]. The highest uptake of the drug in the outer wall region has been reported to result from its transport through the vasorum due to the rheological properties of the drug [45]. Troxerutin inhibits platelet adhesion to the extracellular matrix [18]. This inhibition of platelets adhesion to the extracellular matrix [18] yields an anti-erythrocyte aggregation effect and exerts a favourable

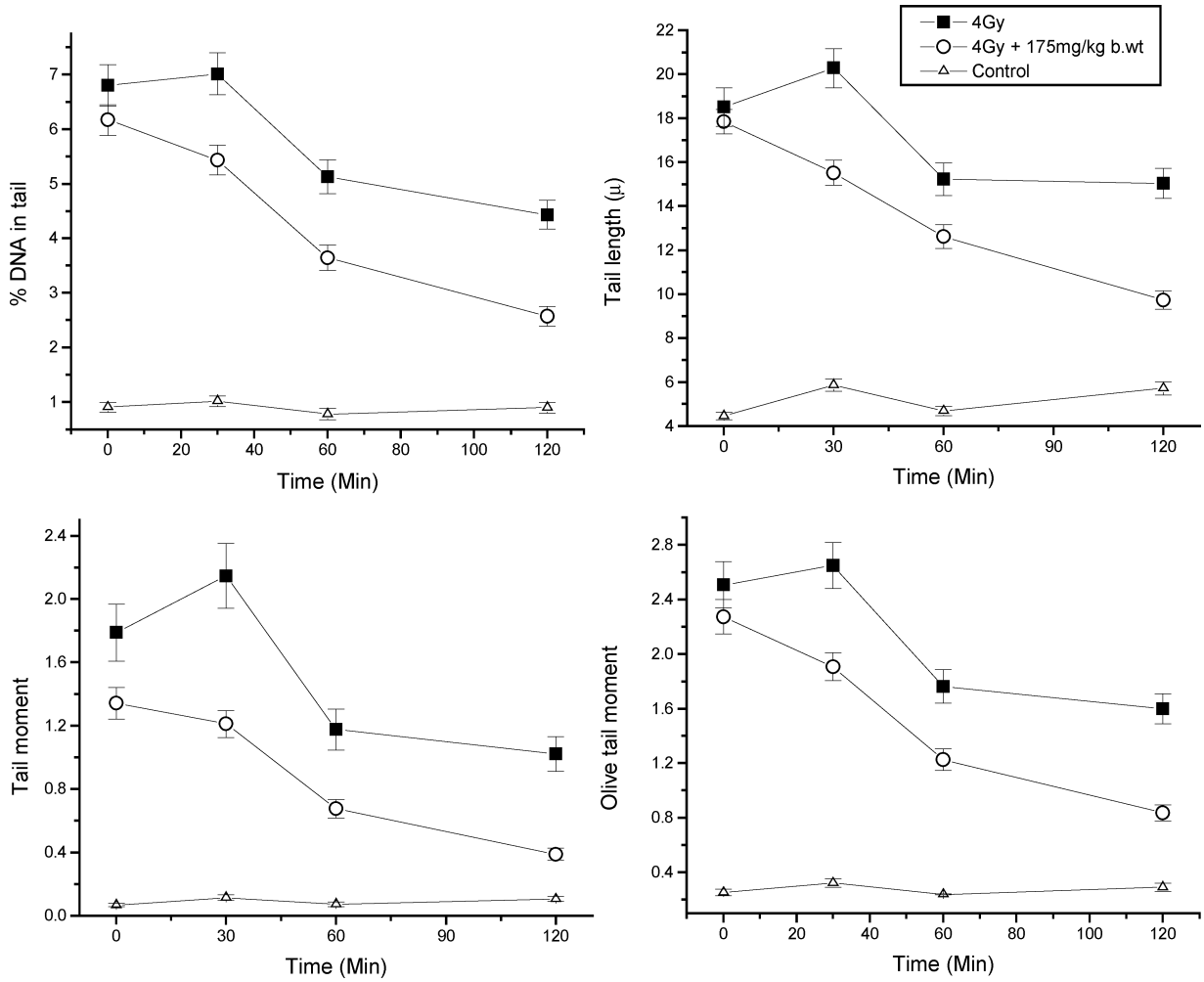

Fig. 6. Effect of troxerutin (175 mg/kg body weight) on repair of murine blood leucocytes DNA strands breaks at different time interval after exposure of 4 Gy γ-radiation in term of decrease in comet parameters: % DNA in tail, tail length, tail moment and Olive tail moment. Each point represent mean ± S.E.M. ($N = 4$).

action on the blood fibrinolytic system [10]. Intramuscular administration of combination of 150 mg of troxerutin and 1.5 mg of carbazochrome was effective in improving hemorrhoidal and post-surgical symptoms following surgery [46].

The present study reveals its use as a safe and effective radio-protector for human application in accident or intentional exposures to ionizing radiation. Recent reports indicate that there is a higher incident of malignancy due to diagnostic exposures to X-rays [47]. This drug could be an ideal radio-protector to be administered prior or after X-ray exposure to protect deleterious genotoxic effects.

Radiation therapy is one of the most common modalities of treatment for human cancers. In order to obtain better tumour control with higher doses of radiation, the normal tissues should be protected against radiation injury. Thus radio-protecting compounds are of importance in clinical radiation therapy [2]. Though a large variety of compounds have shown promise as radio-protector in laboratory studies, few could pass the transition from bench to bed side as most of them failed even before reaching the preclinical stage due to toxicity and side effects. For clinical application of any compound as radio-protector, it would require absolute certainty about the protection factors for tumour and normal tissues to avoid unacceptable clinical risk. The only one compound that is currently used as an adjuvant in radiotherapy for protecting normal tissues is amifostine or WR2721 even though there are reports about contraindications in some cases [48]. Troxerutin has been found to be safe and well tolerated at high concentrations in human subjects. It has been reported earlier that troxerutin protect normal tissues preferentially and does not protect fibrosarcoma-tumour in tumour-bearing animals [28]. Thus the present work suggests that troxerutin could

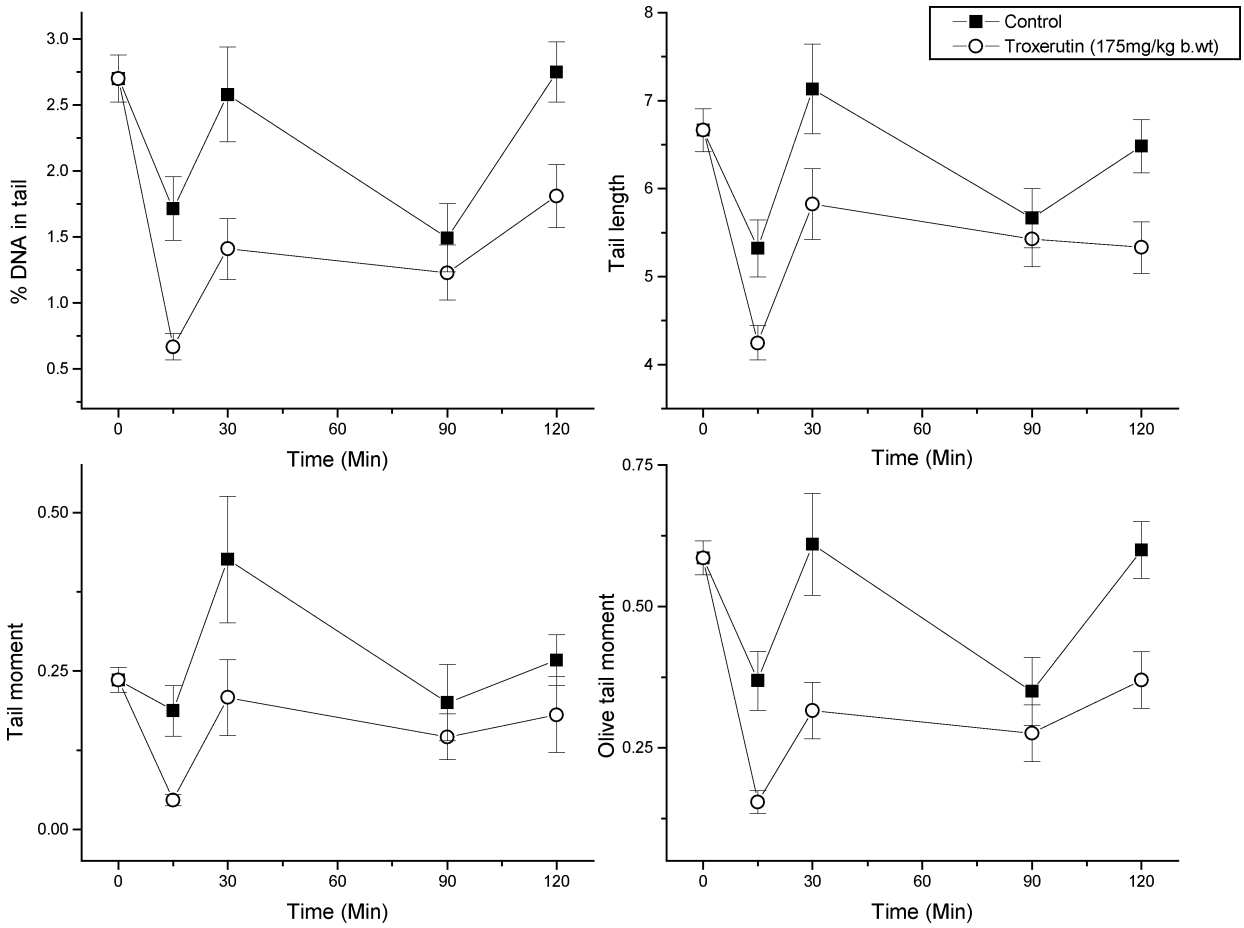

Fig. 7. Effect of troxerutin (175 mg/kg body weight) on murine blood leucocytes DNA strands breaks at different time interval after exposure of 4 Gy γ-radiation in term of decrease in comet parameters: % DNA in tail, tail length, tail moment and Olive tail moment. Each point represent mean $\pm$ S.E.M. ($N = 3$).

be used as an ideal adjuvant in the radiotherapy to protect normal tissues.

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References

1. Arora R, Gupta D, Chawla R, Sagar R, Sharma A, Kumar R, Prasad J, Singh S, Samanta N, Sharma RK: Radioprotection by plant products; present status and future prospects. Phytother Res 19: 1–22, 2005
2. Nair CKK, Parida DK, Nomura T: Radioprotectors in radiotherapy. J Radiat Res (Tokyo) 42(1): 21–37, 2001
3. Weiss JF, Landauer MR: Protection against ionizing radiation by antioxidants nutrients and phytochemicals. Toxicology 189: 1–20, 2003
4. Maisim JR: Chemical radioprotection; past, present and future prospects. Int J Radiat Biol 73: 443–450, 1998
5. Shimoi K, Masuda S, Shen B, Furugori M, Kinae N: Radioprotective effects of antioxidative plant flavonoids in mice. Mutat Res 350(1): 153–161, 1996
6. Rithidech KN, Tungjai M, Whorton EB: Protective effect of apigenin on radiation-induced chromosomal damage in human lymphocytes. Mutat Res (in press)
7. Uma Devi P, Satyamitra M: Protection against prenatal irradiation-induced genomic instability and its consequences in adult mice by Ocimum flavonoids, orientin and vicenin. Int J Radiat Biol 80(9): 653–662, 2004
8. Nayak V, Devi PU: Protection of mouse bone marrow against radiation-induced chromosome damage and stem cell death by the ocimum flavonoids orientin and vicenin. Radiat Res 163(2): 165–171, 2005
9. Rehn D, Golden G, Nocker W, Diebschlag W, Lehmacher W: Comparision between the efficacy and tolerability of oxerutins and troxerutin in the treatment of patients with chronic venous insufficiency. Arzneimittelforschung 43(10): 1060–1063, 1993
10. Boisseau MR, Taccoen A, Garreau C, Vergnes C, Roudaut MF, Garreau-Gomez B: Fibrinolysis and hemorheology in chronic venous insufficiency: a double blind study of troxerutin efficiency. J Cardiovasc Surg (Torino) 36(4): 369–374, 1995
11. Vin F, Chabanel A, Taccoen A, Ducros J, Gruffaz J, Hutinel B, Maillet P, Samama M: Action of the troxerutin on clinical parameters,

plethysmographics and rheologics of venous insufficiency of the lower limbs: a placebo-controled trial. Arteres et Veines 11: 333–342, 1992
12. Vin F, Chabanel A, Taccoen A, Ducros J, Gruffaz J, Hutinel B, Maillet P, Samama M: Double blind trial of the efficacy of troxerutin in chronic venous insufficiency. Phlebology 9: 71–78, 1994
13. Incandela L, De Sanctis MT, Cesarone MR, Laurora G, Belcaro G, Taccoen A, Gerentes I: Efficacy of troxerutin in patients with chronic venous insufficiency: a double-blind, placebo-controlled study. Adv Ther 13(3): 161–166, 1996
14. Boisseau M, Freyburger G, Beylot C, Busquet M: Erythrocyte aggregation and parameters hemorheologic at the insufficiency venous influence of the troxerutin. Arteries et Veins 5(3): 231–234, 1986
15. Wadworth A, Faulds D: Hydroxyethylrutosides. A review of its pharmacology, and therapeutic efficacy in venous insufficiency and related disorders. Drugs 44(6): 1013–1032, 1992
16. Auteri A, Blardi P, Frigerio C, de Lillo L, di Perri T: Pharmacodynamics of troxerutine in patients with chronic venous insufficiency: correlations with plasma drug levels. Int J Clin Pharmacol Res 10(4): 235–241, 1990
17. Glacet-Bernard A, Coscas G, Chabanel A, Zourdani A, Lelong F, Samama MM: A randomized, double-masked study on the treatment of retinal vein occlusion with troxerutin. Am J Ophthalmol 118(4): 421–429, 1994
18. Krupinski K, Giedrojc J, Bielawiec M: Effect of troxerutin on laser-induced thrombus formation in rat mesenteric vessels, coagulation parameter and platelet function. Pol J Pharmacol 48(3): 335–339, 1996
19. Gueguen-Duchesne M, Durand F, Le Goff MC, Genetet B: Effects of troxerutin on the hemorheological parameters of patients with moderate arterial hypertension. Prog Clin Biol Res 280: 401–406, 1988
20. Vanscheidt W, Rabe E, Naser-Hijazi B, Ramelet AA, Partsch H, Diehm C, Schultz-Ehrenburg U, Spengel F, Wirsching M, Gotz V, Schnitker J, Henneicke-von Zepelin HH: The efficacy and safety of a coumarin-/troxerutin-combination (SB-LOT) in patients with chronic venous insufficiency: a double blind placebo-controlled randomized study. Vasa 31(3): 185–190, 2002
21. Marhic C: Clinical and rheological efficacy of troxerutin in obstetric gynecology. Rev Fr Gynecol Obstet 86(2 Pt 2): 209–212, 1991
22. Wijayanegara H, Mose JC, Achmad L, Sobarna R, Permadi W: A clinical trial of hydroxyethylrutosides in treatment of hemorrhoids of pregnancy. J Int Med Res 20: 54–60, 1992
23. Kessler M, Ubeaud G, Walter T, Sturm F, Jung L: Free radical scavenging and skin penetration of troxerutin and vitamin derivatives. J Dermatol Treat 13(3): 133–141, 2002
24. Blasig IE, Loewe H, Elbert B: Radical trapping and lipid peroxidation during myocardial reperfusion injury – radical scavenging by troxerutin in comprision to mannitol. Biomed Biochim Acta 47(8–9): S539–S544, 1987
25. Blasig IE, Loewe H, Elbert B: Effect of troxerutin and methionine on spin trapping of free oxy-radicals. Biomed Biochim Acta 47(10–11): S252–S255, 1988
26. Wenisch C, Biffignandi PM: Effect of bioflavonoids (trihydroxyethylrutin and disodium flavodate) *in vitro* on neutrophil reactive oxygen production and phagocytotic ability to assess by flow cytometry. Curr Med Res Opin 17(2): 123–127, 2001
27. Grotz KA, Henneicke-von Zepelin HH, Khnen R, al-Nawas B, Bockisch A, Kutzner J, Wustenberg P, Naser-Hijazi B, Belz GG, Wagner W: Prospective double-blind study of prophylaxis of radioxerostomia with coumarin/troxerutine in patients with head and neck cancer. Strahlenther Onkol 175(8): 397–403, 1999
28. Maurya DK, Salvi VP, Nair CKK: Radioprotection of normal tissues in tumour-bearing mice by Troxerutin. J Radiat Res 45(2): 221–228, 2004
29. Sreedevi B, Rao BS: Assay of micronuclei in peripheral blood lymphocytes as a biological indicator of radiation dose. Radiat Prot Dosim 50(1): 41–45, 1994
30. Hayashi M, Morita T, Kodama Y, Sofuni T, Ishidate M, Jr: The micronucleus assay with mouse peripheral blood reticulocytes using acridine orange-coated slides. Mutat Res 245(4): 245–249, 1990
31. Singh NP: Microgels for estimation of DNA strand breaks DNA protein crosslinks and apoptosis. Mutat Res 455(1–2): 111–127, 2000
32. Gandhi NM, Maurya DK, Salvi V, Kapoor S, Mukherjee T, Nair CKK: Radioprotection of DNA by Glycyrrhizic acid through scavenging free radicals. J Radiat Res 45: 461–468, 2004
33. Konca K, Lankoff A, Banasik A, Lisowska H, Kuszewski T, Gozdz S, Koza Z, Wojcik A: A cross platform public domain PC image analysis program for the comet assay. Mutat Res 534(1–2): 15–20, 2003
34. Mendiola-Cruz MT, Morales-Ramirez P: Repair kinetics of gamma-ray induced DNA damage determined by single cell gel electrophoresis. Mutat Res 433: 45–52, 1999
35. Belli M, Sapora O, Tobocchini MA: Molecular targets in cellular response to ionizing radiation and implications in space radiation protection. J Radiat Res (Tokyo) 43: S13–S19, 2002
36. Tice RR, Agurell E, Anderson D, Burlinson B, Hartmann A, Kobayashi H, Miyamae Y, Rojas E, Ryu JC, Sasaki YF: Single cell gel/comet assay: guidelines for the *in vitro* and *in vivo* genetic toxicology testing. Environ Mol Mutagen 35(3), 206–221, 2000
37. Nair CKK, Salvi V, Kagiya TV, Rajagopalan R: Relevance of radioprotectors in radiotherapy: studies with Tocopherol Monoglucoside. J Environ Pathol Toxicol Oncol 23(2): 153–160, 2004
38. Olive PL: DNA damage and repair in individual cells: applications of the comet assay in radiobiology. Int J Radiat Biol 75(4): 395–405, 1999
39. Kassie F, Parzefall W, Knassmuller S: Single cell gel electrophoresis assay: a new technique for human biomonitoring studies. Mutat Res 463: 13–31, 2000
40. Hook GJ, Zhang P, Lagroye I, Li L, Higashikubo R, Moros EG, Strube WL, Pickard WF, Baty JD, Roti JR: Measurement of DNA damage and apoptosis in Molt-4 cells after *in vitro* exposure to radiofrequency radiation. Radiat Res 161(2): 193–200, 2004
41. Cordelli E, Fresegna AM, Leter G, Eleuteri P, Spano M, Villani P: Evaluation of DNA damage in different stages of mouse spermatogenesis after testicular X irradiation. Radiat Res 160: 443–451, 2003
42. Singh RK, Verma NC, Kagiya VT: Effect of a water soluble derivative of alpha-tocopherol on radiation response of *Saccharomyces cerivisiae*. Indian J Biochem Biophys 38: 399–405, 2001
43. Marzin D, Phi H, Olivier P, Sauzieres J: Study of the mutagenic activity of troxerutin, a flavonoid derivatives. Toxicol Lett 35(2–3): 297–305, 1987
44. Patwardhan A, Carlsson K, Poullain JC, Taccoen A, Gerentes I: The affinity of troxerutin for the venous wall measured by laser scanning microscopy. J Cardiovasc Surg (Torino) 36(4): 381–385, 1995
45. Carlson K, Patwardhan A, Poullain JC, Gerentes I: Transport et fixation de la troxerutine dans la paroi veineuse. J Mal Vasc 21(Suppl C): 270–274, 1996
46. Basile M, Gidaro S, Pacella M, Biffignandi PM, Gidaro GS: Parental troxerutin and carbazochrome combination in the treatment of post-hemorrhoidectomy status: a randomized, double blind, placebo-controlled, phase IV study. Curr Med Res Opin 17(4): 256–261, 2001
47. Berrington de Gonzalez A, Darby S: Risk of cancer from diagnostic X-rays: estimates for UK and 14 other countries. Lancet 363: 345–351, 2004
48. Tannehill SP, Mehta MP: Amifostine and radiation therapy: past, present and future. Semin Oncol 23(4 Suppl 8): 69–77, 1996