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Radioprotection by tempol: Studies on tissue antioxidant levels, hematopoietic and gastrointestinal systems, in mice whole body exposed to sub- lethal doses of gamma radiation

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Background: Ionizing radiation induces the production of reactive oxygen species (ROS), which play an important causative role in cell death. Whole-body exposure of mice to gamma radiation leads to diminution of tissue antioxidant defense systems; increases the peroxidative damage to membrane lipids and damages the haematopoietic and gastrointestinal systems. Tempol (TPL), a cell membrane-permeable amphilite nitroxide, shown to protect against cell injury caused by ROS was studied for its radioprotective effects. ***Materials and Methods:*** Animals were administered with TPL at doses of 100 or 200 mg/kg body weight *p.o* 10 minutes prior to sub- lethal doses (4 or 6 Gy) of whole body gamma radiation exposure. ***Results:*** Tempol prevented the radiation induced depletion in RBC and total WBC counts, glutathione content in blood and bone marrow cellularity. TPL also protected the tissue antioxidant system and membrane lipids from the radiation-induced damages. An enhanced spleen colony formation and spleen weight recovery were also observed in radiation exposed mice administered with TPL. The compound also protected the epithelial cells of the gastrointestinal tract from the radiation-induced structural alterations. ***Conclusion:*** These preclinical data indicate that TPL may have its potential as a radioprotector during radiation exposure scenarios. **Iran. J. Radiat. Res., 2012; 10(1): 1-10**

Keywords: *Antioxidant defense, radioprotector, hematopoietic system,gastrointestinal mucosa, spleen colony, tempol.*

INTRODUCTION

Total-body exposure to ionizing radiation in humans and animals can result in multiple organ dysfunction as a consequence of damage to the hematopoietic, gastrointestinal or cerebrovascular systems, depending on the total dose of radiation absorbed (1, 2). There remains a need to develop safe and effective radioprotectors which would mitigate the deleterious consequences of radiation exposure in the event of a massive radiological accident, a nuclear terrorist attack, or prolonged space travel (1-5).

Many natural and synthetic compounds have also been found to protect biological systems against radiation induced damage (6 -8). Cyclic nitroxides are stable free radicals stabilized by methyl groups at the α position in six membered piperidine ring structures. The methyl groups confer stability to the nitroxide radicals by preventing radical–radical dismutation. 4-Hydroxy-2,2,6,6-tetramethylpiperidine- *N*-oxyl or tempol (TPL) $C_9H_{18}NO_2$, (scheme 1) is a cell membrane-permeable amphilite nitroxide, a redox cycling agent that can metabolize superoxide anion ($O_2^{\cdot}$) and many other ROS (9-12). The action of nitroxides to metabolize reactive oxygen species is ascribed primarily to cyclic one- or two-electron transfer among three oxidation states: the oxammonium cation, the nitroxide, and the hydroxylamine. Nitroxides undergo a very rapid, one -electron reaction *in vivo* to the corresponding hydroxylamine (13, 14), which has antioxidant activity (9, 10, 15, 16). Tempol protected V79 cells against radiation in a concentration dependent manner (17). Preclinical stud-

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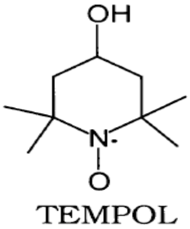
ies in guinea pigs revealed that topical application was effective at preventing radiation-induced alopecia (18, 19). A phase I clinical trial in patients receiving whole-brain radiotherapy suggested that TPL may be effective at preventing radiation- induced alopecia with only mild (grade I and II) toxicity (20). Oral administration of TPL has been shown to prevent the age-dependent rise in blood pressure in the spontaneously hypertensive rats (21) and buthionine sulfoximine induced lipid peroxidation, blood pressure and reduction in cellular levels of glutathione (22). Tempol also protected salivary glands from radiation- induced damage, but did not protect the tumor tissue, suggesting that delivery of the agent prior to irradiation would not alter tumor control (23). Tempol afforded complete protection from the mutagenic effects of hydrogen peroxide and superoxide and was not itself mutagenic (24). It also provided protection against X-ray- and neocarzinostatin-induced mutagenicity and double-strand breaks in DNA (25). Studies using comet assay revealed that TPL and other nitroxides provided significant protection to trout erythrocytes against oxidative damage (26).

In the present study, we explored the protective effects of TPL against depletion of antioxidants, and damages to hematopoietic and gastrointestinal systems in mice whole body exposed to sub-lethal doses of gamma-radiation.



Scheme 1. Chemical structure of tempol.

MATERIALS AND METHODS

Animals

Male Swiss albino mice, 8-10 weeks old and weighing 22-25 g were obtained from the Small Animal Breeding Section, Kerala Agricultural University, Thrissur, Kerala, India. They were kept under standard conditions of temperature and humidity in the Centre's Animal House Facility and provided with standard mouse chow (Sai Durga Feeds and Foods, Bangalore, India) and water *ad libitum*. All animal experiments in this study were carried out with the prior approval of the Institutional Animal Ethics Committee, strictly adhering to the guidelines of Committee for the purpose of Control and Supervision of Experiments on Animals constituted by the Animal Welfare Division of Government of India.

Chemicals

Tempol (TPL) $C_9H_{18}NO_2$, was purchased from Spectrochem Pvt. Ltd., Mumbai, India. Nitro blue tetrazolium (NBT), reduced glutathione (GSH), 5'–5'dithiobis-(2 nitro benzoic acid) (DTNB), EDTA and riboflavin were obtained from Sisco Research Laboratories Ltd., Mumbai, India. TCA (Tri chloro acetic acid) was from Merck Specialties Pvt. Ltd. Mumbai, India. All other chemicals were of analytical grade procured from reputed Indian manufacturers.

Exposure to γ-radiation

Irradiation was carried out using a ^{60}Co-Theratron Phoenix teletherapy unit (Atomic energy Ltd, Ottawa, Canada) at a dose rate of 1.88 Gy per minute.

Administration of TPL

Solution of TPL was prepared in sterile distilled water and animals were administered with TPL by means of oral gavage.

Effect of TPL on γ-radiation induced biochemical and histological alterations in various tissues of whole body radiation (4 Gy) exposed mice

Animals were divided into six groups of three animals each, administered with TPL and exposed to whole body gamma radiation (4 Gy) as detailed below.

Group I- 0.2 ml distilled water + Sham

irradiation, Group II- TPL, 100 mg/kg.b.wt. + Sham irradiation, Group III- TPL, 200 mg/kg.b.wt. + Sham irradiation, Group IV- 0.2 ml distilled water + 4 Gy, Group V- TPL, 100 mg/ kg body weight+ 4 Gy, Group VI- TPL, 200 mg/kg.b.wt. + 4 Gy.

A single dose of TPL (100 mg or 200 mg per kg body weight) was administered to animals 10 minutes prior to the sub- lethal dose of 4 Gy gamma radiation.

a) *Antioxidant status and lipid peroxidation:* After 24 hours of radiation exposure, the animals were sacrificed by cervical dislocation and liver, brain and kidney were excised. Blood was collected by heart puncture into heparinised tubes and analyzed for hemoglobin content by Drabkin's method (27) and GSH content (28). Femurs of the animals were dissected out and bone marrow cells were flushed into phosphate buffered saline (pH 7.4) containing 10% fetal bovine serum. The cells were washed and bone marrow viability was determined by the method of Sredni *et al.* (29). The results were expressed as number of bone marrow cells × 10^6/femur. From the liver, brain and kidney tissues collected, 10% (w/v) homogenates were prepared in ice cold phosphate buffered saline (PBS). These homogenates were analyzed for antioxidant status. Reduced glutathione (GSH) level was measured at 412 nm using DTNB as the substrate (28). Superoxide dismutase activity was determined by the nitro blue tetrazolium (NBT) reduction method of McCord and Fridovich (30, 31). Glutathione peroxidase (GPx) activity was determined by the method of Hafemann *et al.*, (32) based on the degradation of H_2O_2 in the presence of GSH. The concentrations of malondialdehyde (MDA) as indices of lipid peroxidation were assessed according to the method of Buege and Aust (33). Tissue protein was estimated according to the method of Lowry *et al.*, (34) using bovine serum albumin as standard.

b) *Histology of intestine:* At 72nd hour post radiation exposure, animals from different groups were sacrificed. A portion of the small intestine was removed from each group, washed in PBS, and fixed in 10% formaldehyde solution and embedded in wax. Sections were taken and stained with hematoxylin- eosin.

Effect of TPL on different blood parameters, spleen colony formation and spleen weight recovery in whole body radiation (6 Gy) exposed mice

Animals were divided into six groups and administered with TPL, as described before, prior to the sub- lethal dose of 6 Gy whole-body gamma radiation. Blood was collected from the tail vein of each animal every third day (till 12th day post radiation), to heparinised tubes and was analyzed for changes in different peripheral blood parameters viz. RBC, WBC counts and hemoglobin concentration using Mindray BC-2800 Vet auto hematology analyzer. The animals were sacrificed on the 12th day post irradiation by cervical dislocation and the spleen was excised out, weighed and fixed in Bouin's solution and analyzed for colony formations (35-37).

Statistical analysis

The results are presented as mean± standard deviation (SD) of the studied groups. Statistical analyses of the results were performed using ANOVA with Tukey-Kramer multiple comparisons test.

RESULTS

The changes in different antioxidant levels and extent of lipid peroxidation in various tissues of mice exposed to whole body γ-irradiation are presented in table 1. In liver, GSH levels were decreased from 35±2.20 to 22±0.45 nano moles/ mg protein in mice upon exposure to 4 Gy γ- radiation respectively. Administration of TPL prior to radiation exposure maintained the GSH levels to 24.34±8.55 and 29.36±0.67 respectively in TPL100 and TPL200 administered groups. Similar tendency was also observed in other tissues *viz* brain and kidney. As can be seen in table 1, the activity of both SOD and GPx, two of the major enzymes involved in the antioxidant defense mechanism were also found to be decreased after irradiation in all the tissues analyzed and the administration of TPL prior to irradiation in all cases prevented the decrease of both SOD and GPx levels.

Whole body exposure to γ-radiation resulted in an increase in the peroxidation of lipids in different tissues. Table 1 depicts the results on the measurement of peroxidation of lipids in terms of thiobarbituric acid reacting substances monitored as malondialdehyde (MDA) in the brain, liver and kidney of mice exposed to whole body 4 Gy γ-radiation. In liver, the extent of peroxidation of lipids quantified as MDA (nanomoles/ mg protein) were increased from 1.06±0.062 to 4.76±2.35 and administration of TPL prior to radiation exposure showed lower MDA levels, 2.29±0.03 and 1.40±0.15 respectively in TPL100 and TPL200 administered groups. Similar tendency was also observed in other tissues also *viz* brain and kidney where the MDA levels were found to be decreased significantly in the TPL administered animals in a concentration dependent manner.

The protective effect of TPL on the hematopoietic system against deleterious effects of ionizing radiation is evident from the data on bone marrow cellularity (figure 1) and GSH content (figure 2) in blood. The un-irradiated control animals had 15.00 × 10^6 cells/ femur whereas in the irradiated group this dropped drastically to 6.64 × 10^6 cells/ femur. The irradiated animals administered with TPL showed 9.14 × 10^6 cells/ femur and 10.57 × 10^6 cells/ femur in 100 mg/kg.b.wt. and 200 mg/kg.b.wt. groups respectively as compared to the irradiated

Table 1. Changes in antioxidant (GPx, GSH, SOD) and lipid peroxidation levels in 4 Gy whole body irradiated mice (with and without oral administration of Tempol (TPL) $C_9H_{18}NO_2$, (100 or 200 mg/kg body weight, 10 minutes prior to irradiation) in liver, brain and kidney homogenates.

Organ	Treatments	GSH (nano moles/ mg protein)	SOD (units/ mg protein)	GPx (units/ mg protein)	Lipid peroxidation (nano moles/ mg protein)
Liver	0Gy	35±2.20	12.35±1.25	38.41±0.78	1.06±0.06
	TPL 100, 0Gy	27.48±1.94 ^{d}	12.63±2.77 ^{d}	37.86±3.20 ^{d}	1.50±0.90 ^{d}
	TPL 200, 0Gy	29.25±0.71 ^{d,e}	12.81±1.55 ^{d,e}	40.63±6.07 ^{d,e}	0.71±0.29 ^{d,e}
	4Gy	22±0.45	7.5±0.29	20±2.1	4.76±2.35
	TPL 100, 4 Gy	24.34±8.55 ^{d}	11.87±1.13 ^{c}	19.33±0 ^{d}	2.29±0.03 ^{a}
	TPL 200, 4 Gy	29.36±0.67 ^{d,e}	12.13±0.76 ^{c,e}	28.38±5.81 ^{c,z}	1.40±0.15 ^{a,e}
Brain	0Gy	43.73±2.53	15.4±2.86	44.3±1.22	8.4±1.3
	TPL 100, 0Gy	43.16±0.12 ^{d}	14.05±2.15 ^{d}	45.21±4.21 ^{d}	9.54±3.17 ^{d}
	TPL 200, 0Gy	46.72±0.47 ^{d,e}	15.49±1.71 ^{d,e}	39.10±5.53 ^{d,e}	10.28±2.97 ^{d,e}
	4Gy	10.14±1.86	6.3±0.28	22.60±5.38	16.7±2.04
	TPL 100, 4 Gy	31.59±0.13 ^{a}	9.81±1.042 ^{c}	32.06±2.76 ^{c}	14.00±0.53 ^{d}
	TPL 200, 4 Gy	40.8±0.74 ^{a,x}	9.941±0.70 ^{c,e}	36.97±0.33 ^{a,e}	11.57±0.54 ^{b,e}
Kidney	0Gy	41.20±6.10	0.73±0.17	34.71±1.86	3.07±0.60
	TPL 100, 0Gy	36.89±8.78 ^{d}	0.69±0.01 ^{d}	28.62±3.09 ^{d}	3.85±0.36 ^{d}
	TPL 200, 0Gy	35.48±7.78 ^{d,e}	0.68±0.02 ^{d,e}	34.17±2.67 ^{d,e}	3.64±0.63 ^{d,e}
	4Gy	21.94±1.90	0.30±0.00	12.46±2.26	14.10±0.11
	TPL 100, 4 Gy	28.44±1.13 ^{d}	0.43±0.05 ^{d}	16.74±4.65 ^{d}	7.37±0.07 ^{a}
	TPL200, 4 Gy	31.97±1.59 ^{b,e}	0.51±0.03 ^{c,e}	23.56±2.44 ^{a,y}	3.81±1.00 ^{a,x}

(‘a’ indicates $p<0.001$; ‘b’ indicates $p<0.05$; ‘c’ indicates $p<0.01$ and ‘d’ indicates not significant; when compared with the respective control groups; ‘x’ indicates $p<0.001$; ‘ y’ indicates $p<0.05$ and ‘e’ indicates not significant when compared with the respective TPL100 treated groups).

control group. The radiation exposure also brought about drastic drop in blood GSH level and administration of TPL helped to maintain their levels to a considerable extent.

A close microscopic examination of the stained sections of the intestine of radiation exposed animals reveals the altered structures of mucosa and sub- mucosa layers. The irradiated mice exhibited the gastrointestinal damage as crypt epithelial cell necrosis, blunting of the villi and diffused lymphatic and plasmacellular infiltration. The administration of mice with TPL prior to irradiation protected the intestinal epithelial cells from radiation-induced structural alterations as seen in figure 3.

Whole body exposure of mice to gamma radiation resulted in significant depletion of different hematological parameters. Significant increase in total erythrocyte and leukocyte counts, hemoglobin concentration were observed in TPL treated radiation exposed animals as compared to control irradiated animals (figure 4). TPL treated un-irradiated groups showed normal levels of all the hematological parameters (data not shown). Formation of endogenous spleen colonies is an index of hematopoietic stem cell proliferation. TPL administration significantly enhanced the spleen colony formation in animals exposed to a sub-lethal dose of 6 Gy whole body gamma radiation (table 2) in a concentration dependent manner. The control irradiated animals developed an average of 3±0.4 colonies, whereas TPL treated groups developed 17± 0.9 and 26.5± 2.12 colonies for TPL100 and TPL200 respectively. A significant loss in spleen weight was observed in the animals of radiation alone group. On the contrary, the spleen weights were comparatively higher in animals of TPL treated radiation exposed groups.

Table 2. Effect of Tempol (TPL) $C_9H_{18}NO_2$, on spleen colony formation and recovery of spleen weight in mice exposed to a sub-lethal dose of 6 Gy whole-body gamma radiation

Treatments	Number of spleen colonies	Spleen weight, gm
Normal, 0 Gy	0±0	0.235±0.11
Control, 6 Gy	3±0	0.083±0.01
TPL100, 6 Gy	17±9.9 ^{a}	0.111±0.02 ^{d}
TPL200, 6 Gy	26.5±2.12 ^{a,z}	0.174±0.01 ^{a,x}

('a' indicates $p<0.001$; ' b' and 'd' indicates not significant; when compared with the respective control groups; 'x' indicates $p<0.001$; and ' z' indicates $p<0.01$ when compared with the respective TPL100 treated groups).

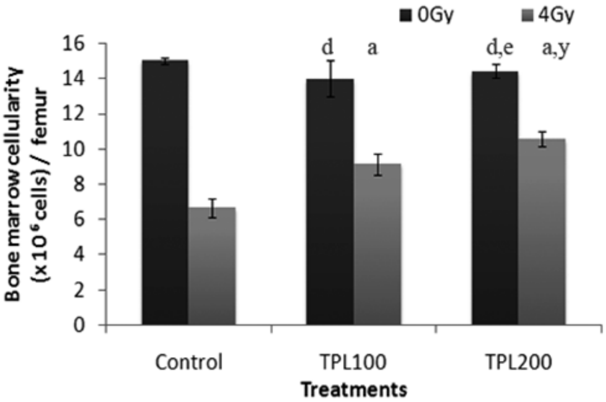


Figure 1. Effect of Tempol (TPL) $C_9H_{18}NO_2$, administration on bone marrow cellularity in mice exposed to 4 Gy whole-body gamma radiation. ('a' indicates $p<0.001$ and'd' indicates not significant; when compared with the respective control groups; 'y' indicates $p<0.05$ and 'e' indicates not significant when compared with the respective TPL100 treated groups).

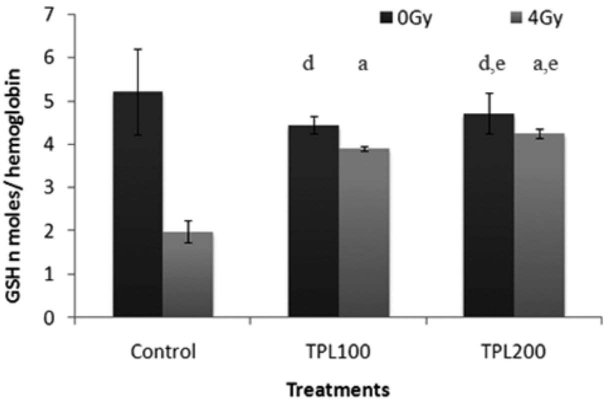


Figure 2. Effect of Tempol (TPL) $C_9H_{18}NO_2$, administration on GSH content in blood of mice exposed to 4 Gy whole-body gamma radiation. ('a' indicates $p<0.001$; and'd' indicates not significant; when compared with the respective control groups; 'e' indicates not significant when compared with the respective TPL100 treated groups).

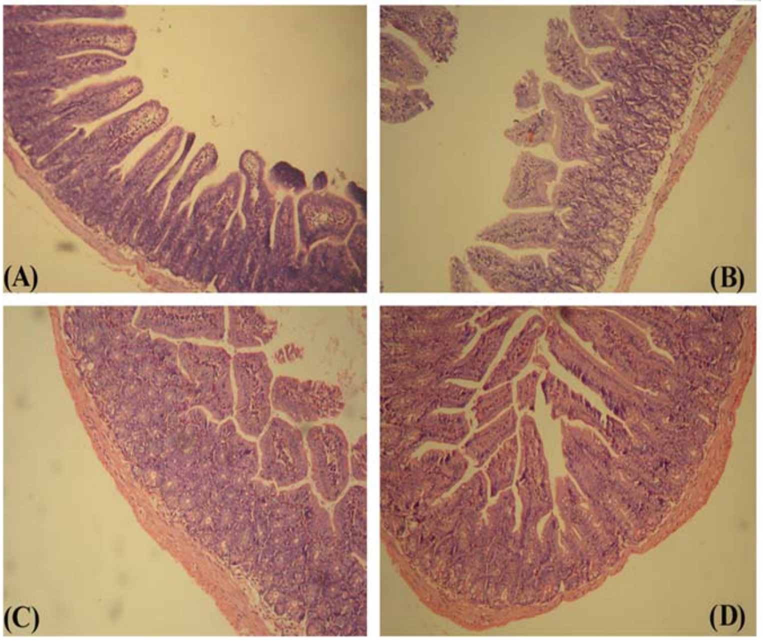


Figure 3. Effect of Tempol (TPL) $C_9H_{18}NO_2$, on gastrointestinal injury of mice upon 4 Gy whole-body radiation exposure. (A) 0 Gy control group; (B) 4 Gy irradiated group showing altered structures of mucosa and sub- mucosa layers. Mice exhibited gastrointestinal damage as crypt epithelial cell necrosis, blunting of the villi and diffused lymphatic and plasmacellular infiltration; Mucosal structure was preserved in the (C) TPL100+4 Gy and (D) TPL200+4 Gy treated groups and pretreatment significantly prevented decrease in villous number and villous height. Magnification X960 (Objective 40X, Eyepiece 10X and Camera zoom 2.4X).

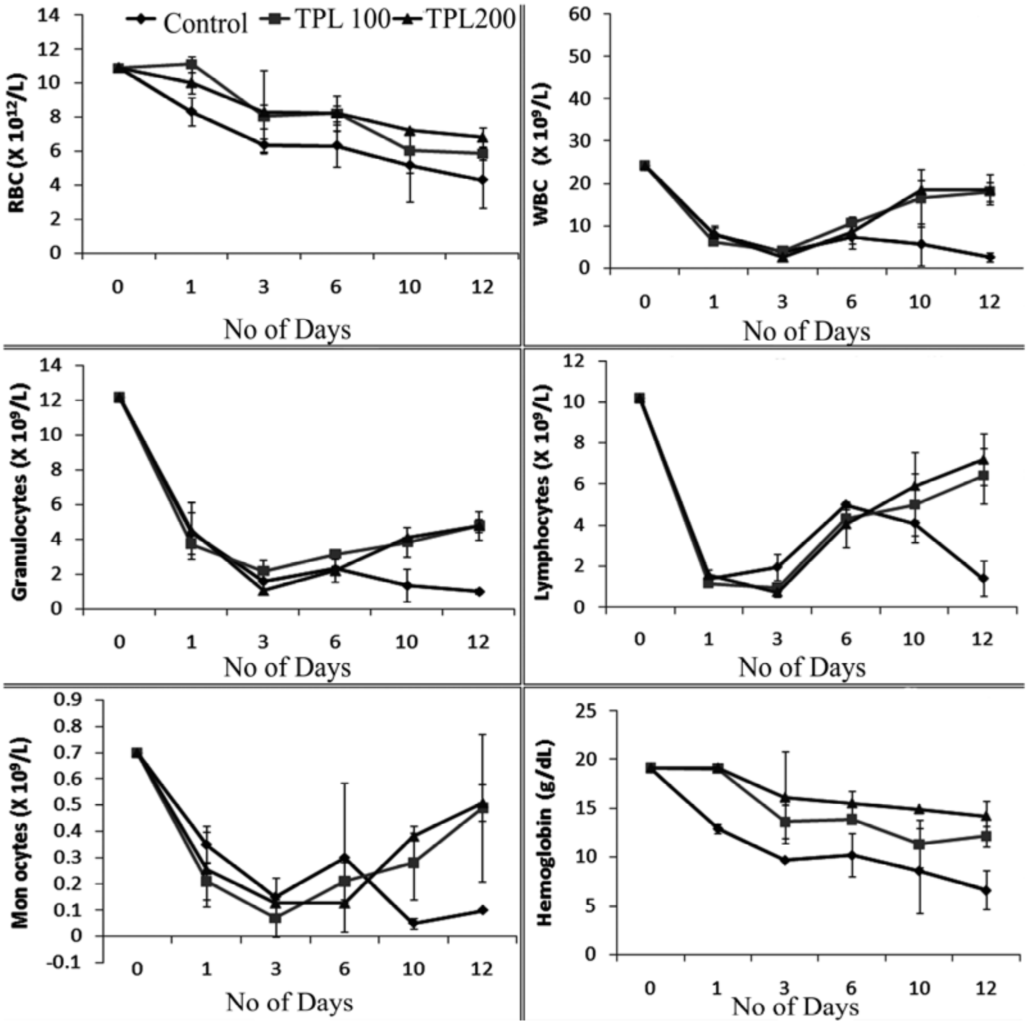


Figure 4. Effect of Tempol (TPL) $C_9H_{18}NO_2$, on different haematological parameters in mice exposed to a sub-lethal dose of 6 Gy gamma radiation.

DISCUSSION

It is well known that most of the damages induced by ionizing radiation to living cells are due to the generation of aqueous free radicals. The body's innate mechanism has many enzymes and non- protein compounds that protect from the free radicals and reactive oxygen species produced inside the body during normal metabolism and also due to external stimuli. Administration of mice with TPL prior to radiation exposure effectively helped to maintain their levels from depletion. The basic effect of radiation on cellular membrane is believed to be the peroxidation of membrane lipids. Lipid peroxidation can be initiated by radiolytic products, including hydroxyl and hydroperoxyl radicals (38). This highly destructive process results in the formation of malondialdehyde (MDA) and alters the total function of the cellular membranes. TPL has been shown to protect lipids (39, 40) and DNA (41-43) from oxidative damages. The present study revealed the efficiency of TPL in inhibiting the radiation-induced lipid peroxidation in different tissues of whole body irradiated mice.

The gastro-intestinal system is one of the major targets for the somatic injuries associated with radiation and chemotherapy. Because of this, radiation-induced gastrointestinal syndrome (RIGS) is an important cause of host vulnerability whether in medical therapeutics or in nuclear accidents or terrorism. RIGS is due in part to the killing of clonogenic crypt cells with eventual depopulation of the intestinal villi (44, 45). Crypt epithelial cells proliferate rapidly and are highly sensitive to cytotoxic agents and irradiation. Loss of this regenerating population of clonogenic cells following irradiation prevents the normal re- epithelialization of the intestinal villi. This impairment leads to varying degrees of villous blunting and fusion, with attenuation and hypertrophy of the villous epithelial cells (46). These changes result in the acute RIGS presenting with malabsorption, electrolyte imbalance, diarrhea, weight loss and death. The late side effects and the sequelae of severe acute intestinal radiation injury include varying degrees of intestinal inflammation, mucosal thickening, collagen deposition, and fibrosis, as well as impairment of mucosal and motor functions (47-49). Tempol protected the intestinal epithelial cells from radiation induced structural lesions to a considerable extent.

Peripheral and lymphoid organ lymphocytes are among the most radiation-sensitive cells (50, 51). Damage to bone marrow is known to be the main cause of death in animals following whole body doses of radiation between about 2 and 10 Gy (52). Radiation death in the mid- lethal dose range is due to impairment of bone marrow hematopoietic function such as leukopenia, erythropenia and thrombocytopenia which will ultimately lead to whole body infection, hemorrhage and even death (53). The protective effect of TPL against radiation injury to hematopoietic tissues was assessed in terms of bone marrow cellularity, blood GSH levels, peripheral blood counts, endogenous spleen colony assay and spleen weight. The decrease in hematological constituents may be attributed to a direct damage by radiation dose and hematopoietic recovery after whole body irradiation is dependent on the presence of spared hematopoietic stem and progenitor cells in the bone marrow (54). The administration of TPL prior to the radiation exposure was associated with significant protective effects against radiation-induced depletion in different hematological parameters. A significant increase was found in spleen weights as well as number of spleen colonies in the TPL and radiation combined groups than the irradiated control group. TPL administration prior to sub- lethal dose of radiation, resulted in higher WBC, RBC and bone marrow cell counts in TPL treated animals compared to the animals of irradiated control group. These findings suggest that prior administration of TPL resulted in protection of hematopoietic stem cells at the time of the radiation exposure, thereby leading to increased recovery of the bone marrow and subsequently the peripheral blood counts.

High exposures to radiation may occur due to accidents or during 'nuclear war'. Radiation also poses a major, un-resolvable risk for astronauts, especially for long-duration space flights (55). The most effective *in vivo* radioprotectors studied so far are effective when administered before irradiation, as they must be present in the system at the time of irradiation. Hence, they can be used only when the eventuality of the exposure is known and are not suitable against unplanned exposures, e.g. accidents, spillage, warfare and terrorist attack. Because conditions of elevated oxidative stress can exist in cells even after irradiation, nitroxides and hydroxylamines can exert protective effects by scavenging secondarily generated ROS resulting from radiation-induced damage (56).

Radiotherapy is being frequently used as part of cancer treatment to achieve tumor control. A major problem associated with cancer radiotherapy is the severe side effects resulting from normal tissue damage. Agents which protect normal tissue against radiation damage can increase the patient tolerance to radiotherapy. Tempol has been shown to differentially protect bone marrow and not tumor cells. Bioreduction of TPL to its corresponding hydroxylamine (which is not a radioprotector) occurred to a greater extent in RIF-1 tumor cells compared to bone marrow (57).

Our present study demonstrates that TPL has capacity to protect the antioxidant, hematopoietic and gastrointestinal systems from radiation induced deleterious effects when administered prior to radiation exposure scenarios. Hence our results coupled with the available literature on the radioprotective effects of TPL suggest that TPL can be a potential candidate for clinical radioprotection.

CONCLUSION

Present study demonstrates that TPL has capacity to protect the antioxidant, hematopoietic and gastrointestinal systems from radiation induced deleterious effects when administered prior to radiation exposure scenarios.

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