

Prevention of Gamma Radiation Induced Anaemia in Mice by Diltiazem

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Radiation/Pro- and normoblasts/Blood Constituents/Erythropoietin/Diltiazem.

Intraperitoneal administration of diltiazem (DTZ), half an hour prior to whole body gamma irradiation (2.5, 5.0, & 7.5 Gy), showed the protection of animals from radiation-induced anaemia. Radiation exposure significantly ($p < 0.001$) reduced the number of pro- and normoblasts in bone marrow and RBC counts, hemoglobin (Hb), hematocrit (Hct), and erythropoietin (EPO) level in blood, but increased myeloid / erythroid ratio. At all the radiation doses, the maximum decrease in these values was noted on the 3rd day, followed by a gradual recovery from the 7th day, but it was not recorded as normal even until the end of experimentation. In animals pretreated with DTZ, these values were measured higher at all the time periods in comparison to corresponding control, and these were almost normal at the last autopsy interval only at 2.5 Gy radiation dose. DTZ maintained the higher erythropoietin level in blood, which acted on bone marrow and spleen colony forming unit for erythroblast (CFU-E), and stimulated such cells to produce RBCs. These results confirm that DTZ has the potency to alter anaemic condition favorably through the protection of bone marrow stem cells, and subsequently it maintains the higher number of pro- and normoblasts in bone marrow, RBC counts, hemoglobin (Hb), hematocrit (Hct) percentage, and erythropoietin level in blood and the lower myeloid/erythroid ratio in bone marrow.

INTRODUCTION

Anaemia is the condition characterized by a reduction in the number of red blood corpuscles (RBCs), the quantity of hemoglobin, and the volume of packed RBCs per 100 ml of blood.¹⁻² Different types of anaemias are known, viz. as (i) anaemia because of blood loss, (ii) aplastic anaemia because of radiation and chemotherapy or the inability of damaged stem cells in the bone marrow to manufacture new red blood cells, (iii) anaemia in which the impaired production of RBCs is due to the deficiency of iron, vitamin B₁₂, protein, ascorbic acid, folic acid, and niacin, and (iv) hemolytic anaemia because of blood loss. Anaemia can be induced experimentally in laboratory animals. Radiation-induced anaemia can be detected by looking at the sample of blood that may show a lack of immature red cells called reticulocytes or a sample of bone marrow that shows an absence of red blood cell precursors. Anaemia is also caused by a low level of erythropoietin. Since kidneys produce this cytokine, such a condition may occur as a result of kidney damage.³

Several attempts have been made to find a suitable radioprotector by taking synthetic compounds like Dimethyl pro-

taglandin,⁴ Sodium tungstate,⁵ Chitosan,⁶ Beer,⁷ Turpentine,⁸ and various plant products such as Liv. 52,⁹ Garlic,¹⁰ Ginseng,¹¹ Spirulina,¹² Ocimum,¹³ and Mentha.¹⁴ Most of these have not yet been clinically applied because of their toxicity at protective dose levels, and a search is still on to find out a potent radioprotector with minimum or no toxicity. Diltiazem is a calcium channel blocker used in cardio-vascular therapy, and it acts by inhibiting the influx of Ca²⁺ through specialized channels into cells and thus influences numerous cell functions.¹⁵ There are some reports on the protective action of calcium channel blockers against radiation.¹⁶⁻²⁴ The present experiment is an attempt to study the protective effect of diltiazem on radiation-induced deleterious changes in blood and bone marrow cells that are responsible for anaemia.

MATERIALS AND METHODS

Animals

Swiss albino mice, 6-8 weeks old and weighing 22 ± 2 g., from a close-bred colony were used for the present study. These animals were maintained under controlled conditions of temperature and light (Light/dark, 10 h/14 h) as per norms laid down by a departmental ethical committee. They were provided standard mice feed (procured from Hindustan Levers Ltd., India) and water *ad libitum*. Tetracycline water once a fortnight was given as preventive measures against infections.

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Irradiation

A cobalt teletherapy unit (ATC-C9) at the Cancer Treatment Centre, Radiotherapy Department, SMS Medical College & Hospital, Jaipur, was used for irradiation. Unanaesthetised animals were restrained in well-ventilated perspex boxes and exposed to gamma radiation at a distance (SSD) of 77.5 cm from the source to deliver the dose rate of 1.04 Gy/min.

Drug

Diltiazem (procured from Dr. Reddy's Laboratory, Hyderabad) was dissolved in double distilled water (DDW) according to the body weight of the animals and injected in mice intraperitoneally (i.p.) as 100 mg/kg body weight.

EXPERIMENTAL DESIGN

Determination of optimum dose of DTZ against radiation

A dose selection of diltiazem was done on the basis of drug tolerance study. For this purpose, various doses of Diltiazem (25, 50, 100, and 200 mg/kg b.wt.) were tested for their tolerance in Swiss albino mice. Thus the most optimum and tolerable dose of diltiazem (100 mg/kg b.wt.) was obtained in our previous study²⁵⁾ and used for further detailed experimentation.

Modification of radiation response

Animals selected for this study were divided into four groups. Animals of group 1 were injected intraperitoneally (i.p.) with double-distilled water or DDW (volume equal to diltiazem) to serve as normal, and animals of Group 2 were given diltiazem (100 mg/kg b.wt.) alone intraperitoneally. Animals of Group 3 received DDW (equivalent to DTZ) and then exposed to different doses of gamma radiation (2.5, 5.0, & 7.5 Gy) to serve as control. Animals of Group 4 were given DTZ (as in Group 2) and exposed after half an hour to different doses of gamma radiation (as in Group 3) to serve as experimental subjects. At least six animals from each group were autopsied at 1/2 day, 1 day, 3 days, 7 days, 14 days, and 28 days of post-irradiation.

Bone marrow smear preparation and counting

Femur bones from the above mice were dissected out and cleaned, their heads were cut, and bone marrows were flushed out and diluted with mice serum with the help of a syringe. Thin films of the cell suspension were prepared on clean glass slides and stained with Leishman's reagent. A total of 500 cells were counted from each slide and the percentage of bone marrow cells were determined in relation to the total cellular count.

Erythropoietin level

Erythropoietin (EPO) level was measured in serum by SRL Ranbaxy Ltd., Mumbai (India). This test was done with the

immulite analyzer kit (Catalog No. LKEPZ) manufactured by Diagnostic Products Corporation, USA.

Hematological study

For hematological parameters, blood was collected from the caudal vein of mice in a vial containing 0.5 M EDTA. Total erythrocyte counts and the haematocrit and hemoglobin values were determined by adopting standard procedures.

Endogenous spleen colony assay (CFU-S)

The endogenous spleen colony assay was done with the slight modification of the method of Till and Mac Culloch.²⁶⁾ Endogenous spleen colony forming units (CFU-S) were determined on day 10 after single whole body irradiation. Briefly, the animals were sacrificed on day 10 after irradiation by cervical dislocation, and their spleens were removed, weighed, and fixed in Bouin's solution, and grossly visible nodules on the surface of the spleen were counted. The nodules observed in the spleens of the irradiated mice were discrete, round, or oval, gray in color, and embedded in the red mass of the spleen. Such nodules were counted by naked eyes.

Statistical analysis

The data were subjected to a Student's *t*-test for comparison between the groups. The values are expressed as mean \pm SEM, and significance levels were set at $p < 0.05$, $p < 0.005$, and $p < 0.001$ levels.

RESULTS

No toxic effects in terms of sickness were observed in the animals treated with DDW (Group 1) and diltiazem (Group 2). All animals exhibited signs of radiation sickness within 2 days after exposure to 7.5 Gy with DDW (Group 3 as control). These symptoms included anorexia, lethargicity, diarrhea, body weight loss, and ruffled fur. The animals started dying from the 7th day and was observed within 30 days of postirradiation. In the animals pretreated with diltiazem (experimental), 87.5% of the animals survived until 30 days after irradiation.

Bone marrow counts

The animals treated with DTZ alone (Group 2) showed no significant change in the number of pro- & normoblasts, and their values were found as nearby normal at all the autopsy intervals. A significant decrease in the number of such cells in group 3 as compared to normal was observed at all the radiation doses (2.5, 5.0, & 7.5 Gy). Maximum decrease was observed on day 3 postirradiation (17.34 ± 0.30 , 13.24 ± 0.48 , & 10.68 ± 0.22 at 2.5, 5.0, & 7.5 Gy respectively). At 2.5 and 5.0 Gy, gradual recovery started from the 7th day, but values could not be recorded near normal even until the end of experimentation. At 7.5 Gy, elevation in counts started from

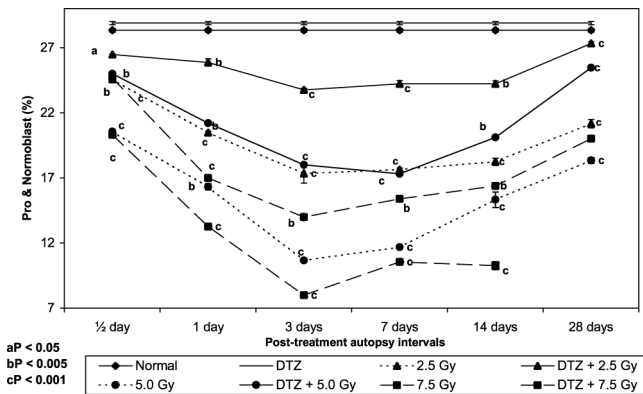


Fig. 1. Variations in pro- and normoblasts (%) in Swiss albino mice after exposure to different doses of gamma radiation with (experimental) or without (control) diltiazem. The values represent mean \pm SEM. The statistic significance was obtained between Normal vs. Control and Control vs. Experimental (^a $p < 0.05$, ^b $p < 0.005$, ^c $p < 0.001$).

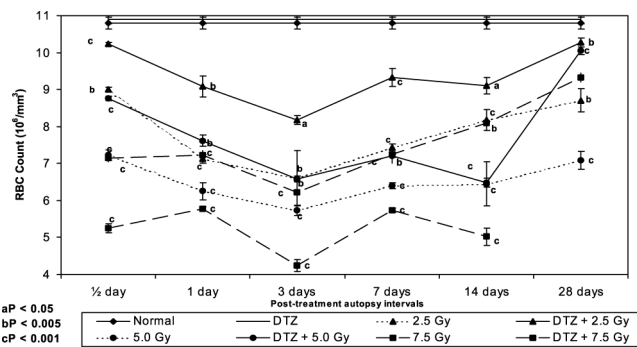


Fig. 2. Variations in RBC counts in Swiss albino mice after exposure to different doses of gamma radiation with (experimental) or without (control) diltiazem. The values represent mean \pm SEM. The statistic significance was obtained between Normal vs. Control and Control vs. Experimental (^a $p < 0.05$, ^b $p < 0.005$, ^c $p < 0.001$).

the 7th day (10.54 ± 0.734) with a gradual decline at the 14th day (10.26 ± 0.526). Pro- and normoblasts number was found to be significantly lesser ($p < 0.001$) at all intervals in this group. In DTZ pretreated animals (Group 4), a significant increase in the counts of pro- and normoblasts was observed in comparison to their respective control (Group 3) at each autopsy interval. Such cells increased from the 7th day, and values were found to be near normal by 28 postirradiation only in the 2.5 Gy radiation dose (Fig. 1).

Hematological studies

Animals of group-2 (DTZ alone) exhibited no significant alteration in hematological values in comparison to the normal.

Total RBC counts decreased the maximum on the 3rd day of autopsy interval (2.5, 5.0, and 7.5 Gy at 6.60 ± 0.74 , 5.73 ± 0.14 , & 4.25 ± 0.16 , respectively) in all radiation doses. At

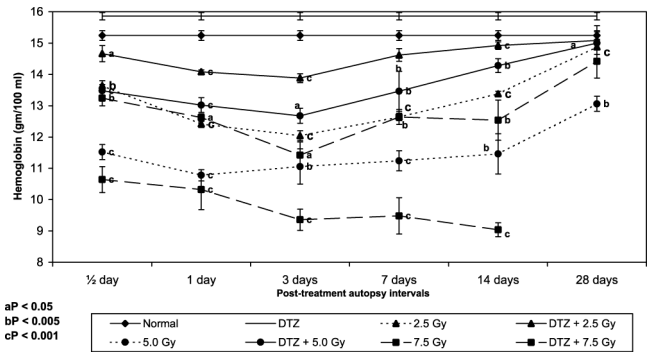


Fig. 3. Variations in haemoglobin concentration in Swiss albino mice after exposure to different doses of gamma radiation with (experimental) or without (control) diltiazem. The values represent mean \pm SEM. The statistic significance was obtained between Normal vs. Control and Control vs. Experimental (^a $p < 0.05$, ^b $p < 0.005$, ^c $p < 0.001$).

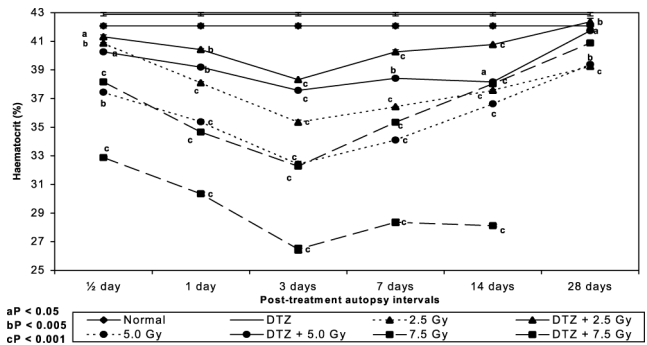


Fig. 4. Variations in haematocrit percentage in Swiss albino mice after exposure to different doses of gamma radiation with (experimental) or without (control) diltiazem. The values represent mean \pm SEM. The statistic significance was obtained between Normal vs. Control and Control vs. Experimental (^a $p < 0.05$, ^b $p < 0.005$, ^c $p < 0.001$).

2.5 and 5.0 Gy, gradual recovery started from the 7th day, but values could not be recorded as normal even until the end of experimentation. At 7.5 Gy, elevation commenced from the 7th day (5.73 ± 0.06) with a subsequent decline at the 14th day (5.02 ± 0.24). RBC counts showed significant decrease ($p < 0.001$) throughout the experiment at all radiation dose levels. Irradiated animals (Group-4, pretreated with DTZ) exhibited a significant increase in RBC counts with respect to control during the entire period of study by attaining the normal value on the last day (i.e., the 28th day), only in the 2.5 Gy radiation dose (Fig. 2).

Hb concentration in irradiated groups (2.5, 5.0, & 7.5 Gy) showed a maximum decrease on the 3rd day (12.04 ± 0.16 , 10.06 ± 0.56 , and 9.36 ± 0.34 , respectively) with a gradual reparation from the 7th day, regaining almost normal level by day 28. In the irradiated group (Group 4, with DTZ), animals showed significantly higher Hb concentration than the respec-

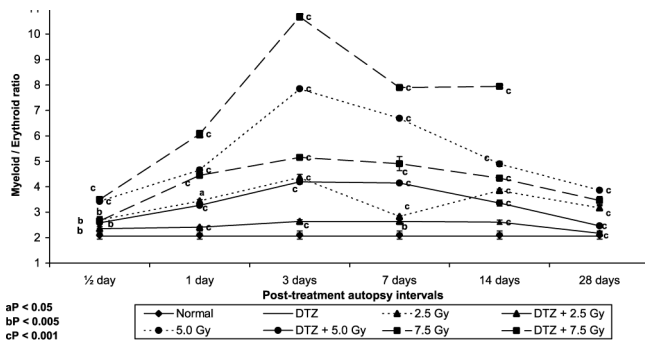


Fig. 5. Variations in myeloid / erythroid ratio in Swiss albino mice after exposure to different doses of gamma radiation with (experimental) or without (control) diltiazem. The values represent mean \pm SEM. The statistic significance was obtained between Normal vs. Control and Control vs. Experimental (^a $p < 0.05$, ^b $p < 0.005$, ^c $p < 0.001$).

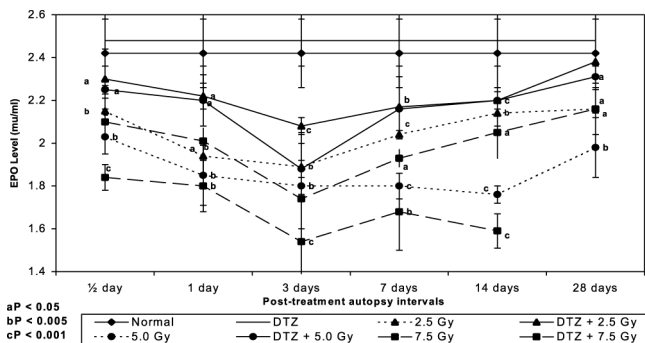


Fig. 6. Variations in erythropoietin level in Swiss albino mice after exposure to different doses of gamma radiation with (experimental) or without (control) diltiazem. The values represent mean \pm SEM. The statistic significance was obtained between Normal vs. Control and Control vs. Experimental (^a $p < 0.05$, ^b $p < 0.005$, ^c $p < 0.001$).

tive control, and almost a normal level was evident at 2.5 Gy radiation dose level (Fig. 3).

Hematocrit (Hct) percentage was found to be significantly lower in all the irradiated groups. The maximum decline was observed on the 3rd day (35.34 ± 0.28 , 32.42 ± 0.16 , and 26.48 ± 0.16 at 2.5, 5.0, and 7.5 Gy respectively). In experimental animals, Hct values were higher in comparison to respective control throughout the period of study (Fig. 4).

Myeloid/erythroid ratio

The animals treated with DTZ showed no significant change in bone marrow myeloid/erythroid ratio in regard to normal. A significant increase in this ratio was observed in control animals (irradiation alone) in comparison to normal at all radiation doses studied (2.5, 5.0, and 7.5 Gy). Maximum increase in control animals was observed on day 3 of postirradiation (4.37 ± 0.12 , 7.85 ± 0.04 , & 10.68 ± 0.10 at 2.5, 5.0, & 7.5 Gy, respectively). At 5.0 Gy, a gradual decrease started

Table 1. Spleen response on day 10 in Swiss albino mice after exposures to different doses of gamma radiation with (Experimental) or without (Control) DTZ.

Radiation dose (Gy)	Group	Spleen colony units (CFU-S)
2.5	Control	None
	Experimental	None
5.0	Control	None
	Experimental	$5.20 \pm 0.33^*$
7.5	Control	$6.66 \pm 0.88^*$
	Experimental	$17.64 \pm 1.76^*$

Significance level: Control vs. Experimental. * $p < 0.001$.

from the 7th day, and values could not be obtained normal till the end of experimentation. At 2.5 and 7.5 Gy, a decline started from the 7th day (2.83 ± 0.04 & 7.90 ± 0.10) with a gradual elevation at the 14th day (3.85 ± 0.06 and 7.94 ± 0.06). The myeloid/erythroid ratio showed significant increases ($p < 0.001$) in all radiation doses. This increase was two times higher in 2.5 Gy- and five times higher in 7.5 Gy-irradiated animals. In DTZ pretreated animals (experimental), a significant decrease in myeloid/erythroid ratio was observed in comparison with the respective control at each autopsy interval, and this became almost normal by 28 postirradiation at 2.5 Gy (Fig. 5).

Endogenous spleen colony assay

The protective effect of DTZ against radiation injury to stem cells was assessed by the endogenous spleen colony assay. No spleen colonies were evident in control and experimental (irradiated) groups at 2.5 Gy or in the control group at 5.0 Gy. However, colonies in the spleen (5.20 ± 0.33) appeared in the experimental group at 5.0 Gy. Similarly, a significant increase in their number over normal was scored in 7.5 Gy control (6.66 ± 0.88), which was significantly enhanced in experimental (17.64 ± 1.76) group (Table 1).

Erythropoietin level

In the present investigation, no significant variation in serum EPO was observed in DTZ-alone treated animals with respect to normal. However, a dose dependent decrease in EPO level was observed in animals, and this decrease was maximum on the 3rd day (1.89 ± 0.16 , 1.80 ± 0.20 , & 1.64 ± 0.22 at 2.5, 5.0, & 7.5 Gy, respectively). Experimental animals exhibited a significant increase in EPO level with respect to control, and these values were nearly normal at 2.5 Gy, but remained below normal at 5.0 and 7.5 Gy doses (Fig. 6).

DISCUSSION

The results from the present study indicate that a pretreatment of DTZ protects the mice from radiation-induced anaemia. The protective effect of DTZ was demonstrated by an increase in colony-forming units in spleen, number of pro-

and normoblasts in bone marrow, various hematological constituents (RBC, Hb, & Hct) and erythropoietin level in peripheral blood. A significant radioprotection was achieved when DTZ was given intraperitoneally as 100 mg/kg b.wt. half an hour prior to irradiation.

Radiation induced concomitant clinical problems of a tendency toward uncontrolled hemorrhage, decreased resistance to infection, and anaemia likewise will vary considerably from as early as 10 days to as late as 6–8 weeks after exposure. A reasonable average time of onset of the clinical problems of bleeding, anaemia, and decreased resistance to infection is 2–3 weeks. This may cause weight loss, hair loss and ultimately death. Following lethal exposures, the marrow may be so damaged that recovery is impossible.²⁷⁾ In the present study, 87.5% survival was observed in diltiazem-pretreated irradiated animals (group 4). Similar results were observed earlier by Floershem,¹⁸⁾ who found that DTZ protects the mice from bone marrow damage and therefore from death from the bone marrow syndrome, allowing survival up to 93% after irradiation. Survival of endogenous spleen colony forming cells²¹⁾ and granulocyte/macrophage colony forming cells (GM-CFC) by diltiazem was also determined at day 10–14, which indicates recovery from radiation damage in bone marrow.²⁸⁾

Normal bone marrow contains cells capable of forming macroscopically visible colonies in the spleens of heavily irradiated animals. Colonies developing in the spleens of mice irradiated with doses between 6 and 8 Gy have been used to study the behavior of colony forming cells *in situ*.²⁶⁾ In the present study, a significant increase in the number of spleen colony forming units was observed in DTZ pretreated animals. Such a number is five times higher in the case of 5.0 Gy-irradiated animals and three times higher in 7.5 Gy-irradiated animals pretreated with DTZ. This shows significant protection of stem cells by diltiazem.

In the irradiated mice, a pool of erythropoietin sensitive cells has been found to be increased. Erythropoietin not only acts on the existing erythropoietin sensitive cells, but also on the stem cells that enter in the process of differentiation,²⁹⁾ enabling these cells to proliferate and differentiate into functioning erythrocytes. CFU-E in the bone marrow is the primary target cell for EPO, and the largest number of EPO receptors are formed at the stage of development between the CFU-E and the proerythroblasts.³⁰⁾ When EPO binds to its receptor, it is rapidly internalized and undergoes endocytosis and degradation. The kidney, especially the peritubular interstitial cells, is the main production site of EPO production.³¹⁾ Kidneys are probably less sensitive to ionizing radiation, but at higher doses (6–8 Gy) they show serious damage,³²⁾ which is further responsible for a decrease in EPO production. In the present study, a significant decrease in serum EPO level was observed in irradiated animals. This decrease was lower in DTZ-pretreated animals, which is because DTZ has specific effects on kidneys. A renal protective effect of DTZ on kid-

neys has been observed by Bakris and Shaikh³³⁾ and Schulman.³⁴⁾ These effects have been divided into those that are primarily mediated by changes in renal blood flow^{35–36)} and secondarily mediated by electrolyte handling,³⁷⁾ whereas some may be renal specific, and others may result from the systemic hypotensive effects that are produced by diltiazem.³⁸⁾ Diltiazem might also protect the tubular necrosis of kidneys through its calcium channel blocking action³⁹⁾ and maintain the normal level of erythropoietin in kidneys and further in blood. Erythropoietin stimulates CFU-E cells in bone marrow and spleen for the formation of reticulocytes⁴⁰⁾ as well as the synthesis of RBC cell membrane proteins and on set of enucleation.^{41–42)}

In the present study, an increase in pro- and normoblasts and in RBCs was observed in DTZ-pretreated animals, which shows that DTZ maintains a high EPO level, and this is responsible for an increase in the number of these cells. Hematocrit is the percentage of whole blood that is made up of cells, and a decrease in its value below normal indicates anaemia. Another measure of anaemia is a decrease in Hb percentage.⁴³⁾ In the present investigation, it has been observed that the Hb level declined significantly following radiation exposure. The minimum value of Hb was obtained on the 3rd day of postirradiation at all the radiation groups (2.5, 5.0, & 7.5 Gy). However, a decrease in the Hb content was observed in the present study as a function of radiation dose. These observations are in accordance with the findings of others.^{44–47)} The decrease in Hb content attributed to a decline in the number of red blood cells. In DTZ-pretreated animals, Hb values were higher at all radiation doses, which shows a significant protection of RBCs by DTZ. An increase in EPO level by DTZ is also directly responsible for an increase in Hb content. The synthesis of Hb begins 12 hrs. after the binding of EPO to its receptor⁴⁸⁾ because of increased iron pool by EPO.⁴⁹⁾ A decrease in Hct percentage is also observed in the present study, which can be attributed to the failure of erythropoiesis, destruction of mature cells, or increased plasma volume.⁵⁰⁾ The decline in hematocrit values is due to decreased erythropoiesis and increased plasma volume. The decrease in the number of pro- and normoblasts as well as RBCs in the present study also supports the view of decreased erythropoiesis as the cause of the decline in hematocrit values. DTZ protects bone marrow and blood erythropoietic cells and maintains the normal percentage of Hct.

Results from the present study suggest that DTZ retains the normal counts of bone marrow stem cells and subsequently hematological counts, which protect the individuals from radiation-induced anaemia.

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