

MODIFICATION OF RADIATION-INDUCED DAMAGE IN MICE BY *ROSEMARINUS OFFICINALIS* EXTRACT (ROE)

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Summary

Intestinal protection in Swiss albino mice against radiation injury by *Rosemarinus officinalis* extract (1000 mg/kg b. wt. orally for 5 consecutive days) was studied after whole-body gamma-irradiation (6 Gy). Crypt survival, villus length, apoptic cells, mitotic figures and goblet cells in jejunum were studied at different autopsy intervals (from 12 hrs. to 30 days) after irradiation. Irradiation produced a significant decrease in crypt survival, mitotic figures and villus length; whereas goblet and apoptic cells showed a significant increase from Sham irradiated animals. Maximum changes in all the parameters were observed on day 3 after irradiation. ROE pre-treated irradiated animals resulted in a significant increase in the number of crypt cells, mitotic figures and villus length; whereas the counts of apoptic and goblet cells showed a significant decrease from respective controls at all the autopsy intervals. Irradiated animals resulted in the elevation in lipid peroxidation and a reduction in glutathione as well as catalase concentration in intestine at 1 hr. post-irradiation. On contrary, ROE treatment before irradiation caused a significant depletion in lipid peroxidation and elevation in glutathione and catalase.

Key words : Radiation, Swiss albino mice, *Rosemarinus officinalis*, Crypt cells, Lipid peroxidation, Glutathione, Catalase.

Introduction

The potential application of radioprotective chemicals in the event of planned exposures or radiation accidents has been investigated from the beginning of the nuclear era. Increased use of radiation for both peaceful and military purposes enhanced the possibility of radiation exposure of living organisms. Some chemicals have been and are being used in mammals for radioprotective activity, however, most of these chemicals could not be used clinically owing to their high toxicity at optimum dose level¹. Hence, it was considered important to explore alternatives to the synthetic compounds that would be radioprotective at non-toxic doses.

Plants have been utilized since time immemorial for curing diseases. Even today, nearly 70% of the world population is dependent on plants for handling their health related problems². Herbal preparations are considered safer and less toxic than synthetic compounds. Therefore, it is obvious that the choices of alternative radioprotectors would include plants and plant products.

Rosemarinus officinalis (Rosemary), belonging to family labiatae, is an evergreen branched bushy shrub, attaining a height of about one meter with upright stems, whitish-blue flowers and dark green leaves. Extract of rosemary relaxes smooth muscles of trachea and intestine, and has choleric, hepatoprotective and mitogenic activity³. The common usage, wide acceptability in human beings, and diverse medicinal and antioxidative properties attributed to Rosemary stimulated us to obtain insight into the radioprotective effect of Rosemary leaves extract in mice exposed to whole – body lethal gamma irradiation

Materials and Methods

Animal care and handling : Adult male Swiss albino mice (6-8 weeks old) weighing 23±2 g from inbred colony were used for the present study. The animals were maintained on the standard mice feed (procured from Hindustan Lever Ltd., India) and water *ad libitum*. Four animals were housed in polypropylene cage containing paddy husk (procured locally) as bedding throughout the experiment. Animal care and handling were performed according to guidelines issued by the World Health Organization (Geneva, Switzerland) and the Indian National Science Academy (New Delhi, India). The Department Ethical Committee has approved the present study.

Preparation of the extract : *Rosemarinus officinalis* was identified by a well known botanist of Botany Department, University of Rajasthan, Jaipur. Leaves of this plant were cleaned, shade dried, powdered and extracted. The extract was prepared by refluxing with double distilled water for 36 hrs (12 hrs. x 3). The cooled liquid extract was concentrated by evaporating its liquid contents so as to make it in powder form. An approximate yield of 22 % extract was obtained. The extract was redissolved in DDW just before oral administration in mice. Henceforth, the extract of *R. officinalis* leaves will be called as ROE.

Selection of optimum dose : Dose selection of *Rosemarinus officinalis* (ROE) extract was done on the basis of our previously conducted drug tolerance and animal survival study⁴. Various doses of ROE (100, 200, 400, 800, 1000, 1500 or 2000 mg/kg b.wt.) were tested against gamma irradiation (8 Gy) for radiation sickness and mortality. Optimum dose (1000 mg/kg b.wt.) thus obtained was used for experimentation in details.

Radioprotective effect of ROE

The mice were divided into four groups : Animals in Group-1 were administered orally with DDW (volume equal to ROE) to served as vehicle treated while animals in Group-2 were given ROE orally at a dose of 1000 mg/kg b.wt. Animals of Group-3 received an equal volume of DDW (as in Group-1) orally for 5 consecutive days, once daily. This group served as irradiated control. Animals in Group-4 received ROE (as in Group-2) and served as experimental.

Half an hour after the last administration of DDW or ROE on the day 5th, animals of all groups were whole-body exposed to 6 Gy of ⁶⁰Co gamma radiation (Theratron Atomic Energy Agency, Canada) in a specially designed well ventilated acrylic box. A batch of 12 animals was irradiated each time to 6 Gy radiation at a dose rate of 0.85 Gy/min., at a source-to-animal distance (midpoint) of 77.5 c.m. The animals from the above Groups were autopsied at 12 hrs., 24 hrs., 3, 5, 10, 20 and 30 days of post-irradiation. A part of small intestine (as jejunum) was taken out from each autopsied animal and examined for histopathological and biochemical alterations after routine procedure.

Biochemical determinants

The following biochemical determinants were carried out in survivors of both DDW + irradiation and ROE + irradiation groups at 1 hr. post-exposure.

Glutathione (GSH) assay : The hepatic and intestinal level of glutathione (GSH) was determined by the method of Moron *et al*⁵. The GSH content in blood was measured Spectrophotometrically using Ellman's reagent with 5-5', dithiobis-2-nitrobenzoic acid [DTNB] as a colouring reagent, according to the method of Beutler *et al*⁶. The absorbance was read at 412 nm using a UV-VIS Systronic Spectrophotometer.

Lipid peroxidation assay : The lipid peroxidation (LPx) level in liver, intestine and serum was measured by the assay of thiobarbituric acid reactive substances (TBARS) using the method of Ohkhawa *et al*⁷ in which the absorbance was read at 532 nm using a UV-VIS Systronic spectrophotometer.

Catalase estimation : The catalase activity was measured in intestinal cells by catalytic reduction of hydrogen peroxide using the method of Abei⁸.

Results

Radiation sickness and mortality

Animals of 6 Gy irradiation (Group-3) resulted in radiation sickness within 10 days after exposure. The symptoms included reduction in food and water intake, diarrhea, lethargy, emaciation, epilation and ruffling of hairs. Daily administration of the ROE for 5 consecutive days did not cause any radiation-induced mortality. ROE administration delayed the appearance and radiation sickness like reduction in the diarrhea, irritability, lethargy and food and water intake.

Intestinal parameters

Crypt cells/ Crypt section

Maximum decrease in the number of crypt cells was observed on day 3rd in both control and experimental groups. Day 5th onwards, there was an increase in the number of cells in the animals of both the groups. In control, these cells could not attain the normal value even till the last autopsy interval; whereas in experimental, the number of crypt cells was found within normal range (Table-1).

Mitotic figures/ Crypt section

The frequency of such figures was reduced to nearly half in comparison to normal on 24 hrs. post-irradiation. The maximum decrease was evident on day 3 post-treatment in both control and experimental groups. It was followed by an increase in number on day 5th with a continuous elevation till the end of the experiment, but in control it could not reach the normal level; whereas in experimental, these figures were counted almost normal (Table-1).

Apoptotic cells/ Crypt section

The maximum number of dead cells was noticed on day 3rd after irradiation in both control and experimental groups, but afterwards the number of these cells decreased progressively with the advent of post-irradiation time. However, the frequency of such cells was found to be significantly lower in ROE pre-treated irradiated group (Table –1).

Goblet cells/Vilus section

A significant increase in the number of goblet cells was observed in both the groups on day 1. Day 3 onwards, these cells started to decrease till the last autopsy interval. The number of goblet cells was found to be still higher and did not reach to normal level even on day 30th in control group; whereas in the experimental, these cells were scored almost normal (Table-1).

Villus length

The maximum reduction in the villus height was recorded on day 3rd in both control and experimental groups. At later intervals, mucosa exhibited the sign of recovery that was observed in the form of an increase in height of villi in these groups. In experimental group, recovery from lesions was faster and almost a normal length was measured on day 30th with a significant difference to control (Table-1).

Biochemical determinants

No significant alterations in the intestinal, hepatic and blood GSH contents were observed between normal and ROE treated animals. However, a statistically significant ($p < 0.001$) decrease in GSH was evident in control animals.

Experimental animals showed a significant increase in GSH content (blood, liver & intestine) with respect to control, but the values remained below normal (Fig. 1). Administration of ROE alone before irradiation did not alter the lipid peroxidation. Exposure of animals to gamma radiation increased LPx in Group-3 while the same was significantly reduced in the ROE + irradiation group (Fig. 2). Treatment with *Rosemarinus officinalis* alone (Group-2) elevated catalase level significantly in the non-irradiated group while radiation exposure significantly reduced the catalase concentration (Group-3). A significantly increase in catalase activity was observed in animals, administered ROE before irradiation (Group-4) (Fig. 3).

Fig. 1 Glutathione (GSH) level in blood, liver and intestine after 6 Gy gamma irradiation with (experimental) or without (control) ROE

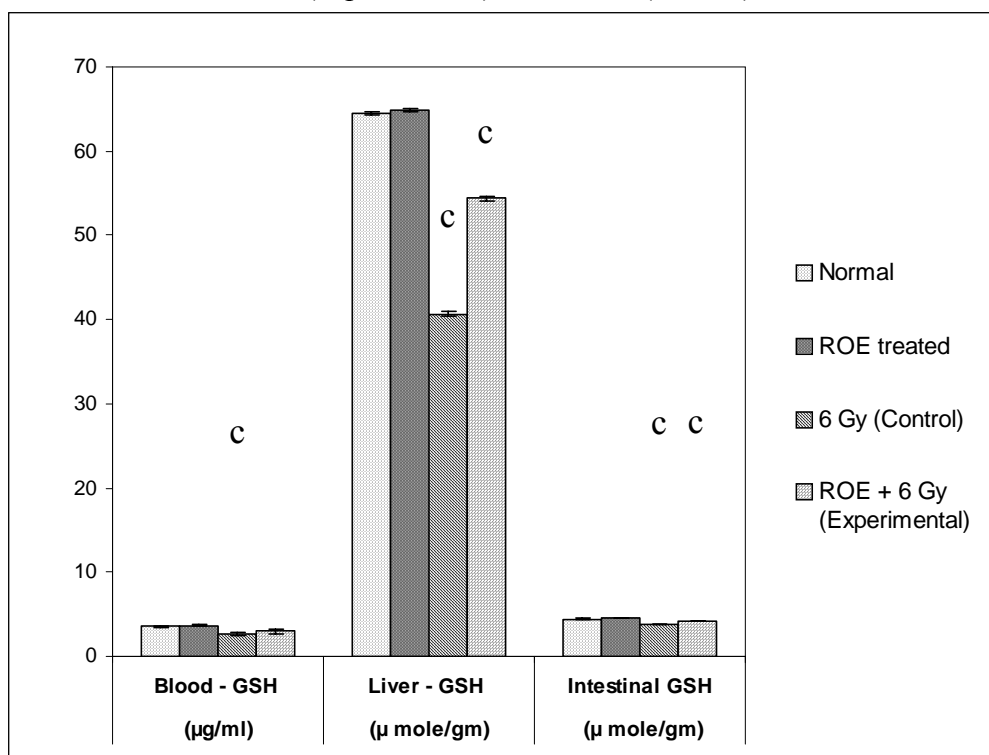


Fig. 2 Lipid peroxidation (LPx) level in blood, liver and intestine after 6 Gy gamma irradiation with (experimental) or without (control) ROE

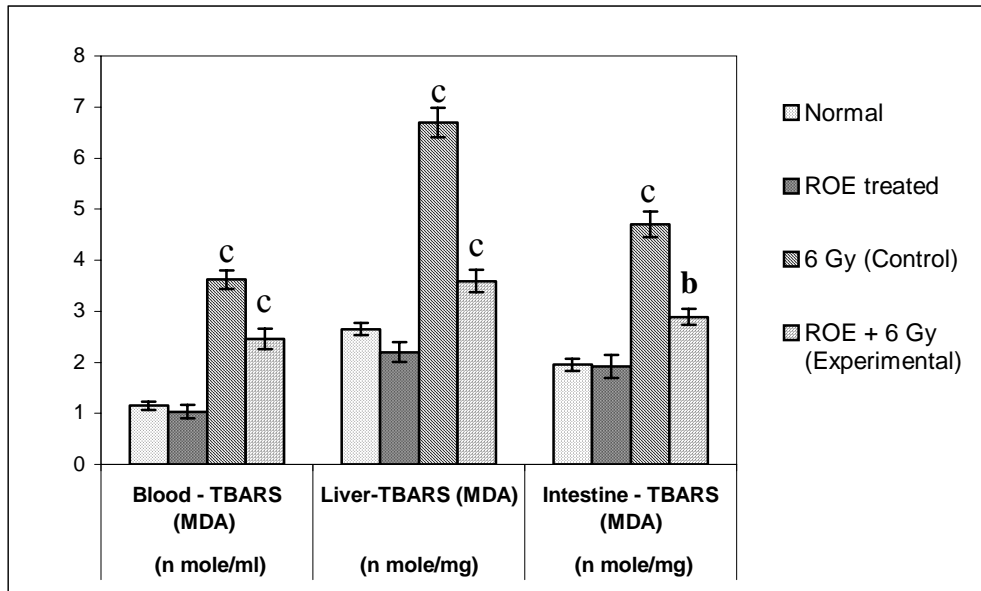
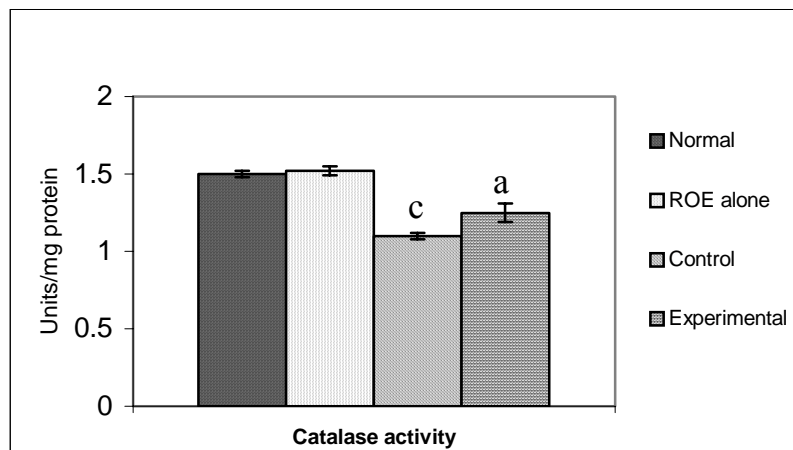


Fig. 3 Catalase activity in intestine after 6 Gy gamma irradiation with (experimental) or without (control) ROE



Significance level : a = $p < 0.05$, b = $p < 0.005$, c = $p < 0.001$

Discussion

Irradiation of animals to 6 Gy gamma rays, in the present study, resulted in radiation sickness within 3-5 days after exposure. The symptoms included reduction in the food and water intake, weight loss, diarrhea, ruffling of hairs and irritability. The similar symptoms have been observed in mice after gamma irradiation by others also^{9,10}.

Whole-body irradiation primarily affects rapidly proliferating germinal epithelium, gastro-intestinal epithelium, bone marrow and spleen progenitor cells. While the germinal epithelium does not have a life supporting function for the exposed individual, but the bone marrow, spleen progenitor cells and gastro-intestinal epithelium cells are crucial for the sustenance of life, and any damage to these cells will impair normal physiological host defense processes drastically, causing an adverse impact on survival.

The gastro-intestinal tract is a cell renewal system and consisting of cells with different radiosensitivity. According to Withers and Elkind¹¹ crypt cells are more sensitive than villus epithelial cells as indicated by the presence of more severe pathological lesions in crypts than those of villi at early intervals. Reduction in villus height and crypt cell population, suppression of mitosis and increase in number of goblet and dead cells were the major pathological changes after exposure to 6 Gy of gamma rays in the intestinal mucosa in this study.

In the present study, the maximum decrease in the number of crypts cells was noted on day 3 in the animals of control group after exposure to 6 Gy of gamma radiation. Similar results were also reported by others¹²⁻¹⁸ who observed the decreased number of these cells in crypt region after irradiation. It has been suggested earlier that reduction in the total cell population of the crypt is due to an early and marked decline in DNA synthesis in the crypt cells, cell death and movement of cells up to villus in the absence of replacement by cellular proliferation¹⁹. The decline in the number of crypt cells was due to a sharp diminution of mitotic activity in the crypts. After exposure to ionizing radiation, rapidly replicating crypt transit cells either undergo apoptosis or cease replication²⁰.

A decrease in number of mitotic figures and minimum number of such dividing cells were recorded on day 3 after irradiation when a cell is exposed to small dose of radiation between late or very early prophase then mitosis stops or cell reverts to an early prophase²¹.

Ionizing radiation affects the cells in all the phases but degree to which these are affected is dependent on the phase (G_1 , S_1 , G_2 or M) in which they were at the time of irradiation^{22,23}. Decline in the number of mitotic cells at early intervals may be attributed to a block of cells in G_2 phase of cell cycle and to prolongation of mitotic process^{24,21}.

The number of apoptic cells/crypt section was found to be increased at the early intervals after irradiation. These cells appeared on 12 hrs. and their number reached to maximum on day 3rd. After this, there was a gradual decrease but it could not restore to normal level until the last autopsy interval.

The high radiosensitivity of dividing and undifferentiating cells might have attributed the death of cells in crypt region. These findings are in close agreement of Devik²⁵ who suggested that most of the early mitosis contain cytological injury due to high dose and might give rise to nonviable degenerative cells.

In the present study, the number of goblet cells has increased on day 1 but thereafter their number declined continuously up to last autopsy interval. The elevation in glycogen content after irradiation may result in an increase in the number of goblet cells. These result are in close agreement with finding of Singh^{26,15,16}.

Villus height was found to be reduced from 12 hrs. and maximum reduction was noted on day 3 after exposure to gamma rays. Loss of epithelial cells from the villi is the basic reason of reduction in height. Similar type of changes were also observed as a result of radiophosphorous administration as well as external irradiation. Thus, the damage in the proliferative crypt region compartment results in the reduction of villi height and deformed tips²⁷.

Administration of ROE, prior exposure to 6 Gy of gamma ray, reduced the severity of radiation induced various quantitative changes in the intestinal mucosa. Though, there was a reduction in crypt cell population, mitotic figures, villus height and increase in number of goblet and dead cells in the ROE treated irradiated animals but changes in these parameters were less pronounced as compared to that of the control animals.

Both the height and structure of the villus were less affected in ROE protected irradiated animals. This could be possible due to the less mitotic death as well as early and least migration of cells from protected crypts to villi before they become denuded.

In the ROE pretreated irradiated animals, the number of goblet cells and dead cells was found to be increased significantly on 12 hrs. which followed a decline continuously till the last autopsy interval. However, the increase in the number was significantly lesser as compared to that of the respective control animals. Similar findings were also observed by others^{12,15}.

The number of crypt cells/crypt section in experimental animals remained at a higher level because of low cell death and increased rate of mitosis as compared to the animals of their respective control group. These results are in good agreement with the observations of Prasanna and Uma

Devi²⁸. The higher number of mitotic figures in experimental animals than the control was in accordance with the studies of Saxena and Goyal²⁹ and Samarth *et al*¹⁵.

Drug metabolizing and oxidation reduction systems in the epithelium of the small intestine represent a first line of defense against ingested toxins. Epithelial cells in the intestine have a characteristically rapid turn over rate that appears to be essential to protect it against injury from exogenous agents^{30,31}. GSH is a versatile protector and executes its radioprotective function through free radical scavenging, restoration of the damaged molecules by hydrogen donation, reduction of peroxides and maintenance of protein thiols in the reduced state³².

In the present study, it was observed that ROE treatment significantly lowered the radiation induced lipid peroxidation in terms of malendialdehyde. Inhibition of lipid peroxidation in biomembranes can be caused by antioxidants. It has been shown that more tocopherol is needed in the membranes to protect polyunsaturated fatty acids (PUFA) against radiation-induced lipid peroxidation³³.

Catalase (CAT) is an enzyme, present in most cells, that catalyses the decomposition of hydrogen peroxide to water and oxygen. CAT is a heme containing protein and it is found to be important in the inactivation of many environmental mutagens³⁴. ROE has elevated the cellular catalase levels accompanied by an arrest of radiation induced depletion of catalase in cells. This may be helpful to relieve the oxidative stress of the cells.

Conclusion

Thus, the results from the present study suggest that the pretreatment of Rosemary extract protects mouse jejunum against the radiation induced reduction in villus height, cryptl cells and mitotic figures/crypt section and increase in goblet cells/villus section and dead cells/crypt section in jejunum of mice. ROE pretreatment protects radiation induced biochemical damage by inhibiting the GSH and catalase depletion and decreasing the LPx level.

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