Research Article

Changes in Spleen Cell Populations in Total Body ^{60}CO-Gamma Irradiated Mice and Their Modification by SBL-1: Implication in Radiation Protection

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ABSTRACT

One time treatment with standardized extract from leaves of *Hippophae rhamnoides* L. (coded as SBL-1), 30min before irradiation with lethal dose (10 Gy) of ^{60}Co-gamma rays rendered >90% radioprotection to mice population. The present study was planned to examine the effects of SBL-1 on mouse spleen cell populations in whole body lethally irradiated mice. All non-SBL-1 treated irradiated (10 Gy) mice died by day 12. In comparison to untreated controls, the irradiated mice showed decreases in (i) spleen/body weight (b.w.) ratio; and (ii) spleen cells count on all observation days. Treatment of mice with SBL-1 before irradiation countered the radiation induced changes; the spleen/b.w. ratio; cell numbers (natural killer cells, macrophages and megakaryocytes) were not significantly ($P \leq 0.05$) different than the untreated controls at day 15. This study demonstrated that SBL-1 treatment before irradiation could restore the cellularity of spleen to near normal by day 15 and therefore was one of the important mechanisms of radioprotective action by SBL-1.

Keywords: *Hippophae rhamnoides*, Spleen, Ionizing radiation, macrophages, natural killer cells.

INTRODUCTION

The haematopoietic system is highly radiosensitive and the severity of damage is radiation dose dependent. Spleen is a part of haematopoietic system and performs multiple important physiological functions such as mechanical filtration of cellular debris; removal of older erythrocytes as well as damaged and aberrant cells from the circulation; recycling of iron; regulation of immune system; and fetal hematopoiesis. It comprises two highly organized compartments (red pulp and white pulp). The red pulp of the spleen removes the aged and damaged RBCs, and blood-born microorganisms from the circulating blood. In this process macrophages, monocytes, megakaryocytes and granulocytes (Eosinophils, Basophils and Neutrophils), participate actively. Monocytes reside in the red pulp cords of the spleen and are in far greater number than the total number of monocytes in circulation. They can be rapidly mobilized to leave the spleen and participate in combating infections.[1]

The immediate exposure of dead apoptotic cells to the antigen presenting cells (macrophages/ monocytes) takes place in the spleen.[2] Macrophages of red pulp are associated with the reticular cells and carry out phagocytosis of aged erythrocytes which are stuck in the cords of the red pulp. This helps in recycling of iron.

Macrophages present in the white pulp of spleen help in scavenging the blood-born debris, clearing of both dead pathogens and dead neutrophils that infiltrate into the spleen.

The granulocytes such as neutrophils are present in the red pulp and form an important component of innate immune response; eosinophils participate in defence against parasitic infections; and basophils are known for their role in allergic inflammations.

Lymphoid progenitor cells form an important component of white pulp of spleen and give rise to various sub-populations of lymphocytes [B-lymphocyte, T-lymphocyte and Natural Killer (NK) cell]. The B-lymphocytes or B-cells reside in the marginal zone (MZ) as well as in the follicular zone (FZ) of white pulp of spleen. B-cell, on activation, get differentiated into plasma cells, which secrete antibodies to fight against infections. The MZ B cells are the first line of defense against systemic blood-borne antigens that are trapped in the spleen after entering the circulation.[3] The B-cells present in the FZ express high levels of IgM, IgD and CD 23. Contrary to the MZ B cells, the FZ B cells, are dependent on T-cell to promote effective primary immune responses. The T lymphocytes or T cells, reside in T-cell zone of spleen (also known as the periarteriolar lymphoid sheath, PALS). These cells, on activation, differentiate into cytotoxic T cells (kill the infected cells) and helper T cell that activate other cells such as B cells and macrophages. The third and the largest lineage of lymphoid cells, NK cell, are cytotoxic granular lymphocyte and form first line of defence against intracellular pathogens. They are also able to recognize and kill some abnormal cells such as tumour cells and virus-infected cells.

Low LET- ionizing radiation (IR) are deeply penetrating and injure multiple organs of the body by direct deposition of energy as well as by generation of free-radicals and reactive oxygen species. Whole body exposure to lethal dose (10 Gy) of ^{60}Co-gamma radiation is known to cause multi-organ disfunction syndrome or multi-organ failure, ultimately leading to death. Suppression of immune response has been observed at

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early stages after irradiation, thereby making the irradiated animals susceptible to pathogens and causing multiple infections.

Development of a safe and non-toxic radiation countermeasure has remained a global challenge despite decades of rigorous research. The single molecule, synthetic drugs, have failed due to several severe side effects (e.g. nausea, vomiting, diarrhoea, hypotension, hypocalcaemia, nephro and neuro toxicity) at clinically effective doses.[4-7] Therefore, more recently, the herbal preparations are gaining more attention of researchers because of their relatively less toxic nature (non-toxic to human).[8,9] Large number of plants such as *Gingko biloba*, *Centella asiatica*, *Ocimum sanctum*, *Panax ginseng*, *Amaranthus paniculatus*, *Emblica officinalis*, *Phyllanthus amarus*, and *Aphanamixis polystachya* have shown radioprotective properties. However, these preparations have been successful at low doses of radiation only.[10] Development of radiation countermeasure at lethal doses (10 Gy) has remained a global challenge.

Hippophae rhamnoides L. (family *Elaegnaceae*), also known as seabuckthorn is a spiny, hard, deciduous shrub that attains 2-4 m height in natural habitat and has long and narrow leaves. It can withstand temperature from -43 ^{0}C to -40 ^{0}C. It is typically cultivated at dry sand areas that are native to Europe and Asia, while it has also been introduced in North and South America. *Hippophae* leaves contain many bioactive compounds[11] and are rich source of natural antioxidants[12] ,flavones[13] and flavonoids[12], vitamins A, C, E and K[14] and tannins.[15] In traditional medical system, it has been recommended for the treatment of hypoxia[16], liver cirrhosis[17], gastric ulcers, cardiovascular diseases, burns and wounds, hepatic injuries and neoplasms.[18]

A standardized and well characterized extract from leaves of *Hippophae rhamnoides* (coded as SBL-1) was prepared (under patenting), which showed significant radioprotective properties. A single dose of SBL-1 [(30 mg/Kg, body weight (b.w)] administered to mice 30min before irradiation to lethal dose of ^{60}Co-gamma rays (10 Gy), rendered survival of >90% populations in comparison to the zero survival in non-SBL-1 treated irradiated (10 Gy) mice populations.[19]

All non-drug (SBL-1) treated irradiated (10 Gy) animals had died after day 12. The treatment with SBL-1 was found to protect various body tissues and also counter the radiation induced behavioural changes[20]; histological changes in kidney[21]; mutagenic effects[22,24]; inflammation and immunosupp恶ression[23] and oxidative stress.[24]

Spleen acquires an important immuno-protective role in the body in addition to other important multiple vital functions. Inadequate information is available in the literature about the differential radiosenstivity of various spleen cells. The present study was planned to investigate the differential radiosenstivity of various types of spleen cells and its modification by SBL-1 when administered to mice at radioprotective dose before total body ^{60}Co-gamma irradiation (10 Gy).

MATERIALS AND METHODS

Reagents

RPMI 1640 medium (with L-glutamine and 25mM HEPES buffer), fetal bovine serum (FBS), penicillin, streptomycin and sodium bicarbonate, ammonium chloride, potassium hydrogen carbonate, EDTA and Giemsa stain were purchased from Hi-Media Laboratory Ltd. (Mumbai, India), DPX and Immersion oil were purchased from Sigma Chemical Co., St. Louis, MO. All other chemicals used were of analytical grade.

Plant extract (SBL-1) and its characterization

The plant extract was prepared and characterized as per procedure described earlier. Briefly, the fresh green leaves of *Hippophae rhamnoides L.* (F. *Elaegnaceae*), identified by ethno-botanists and confirmed as *Hippophae rhamnoides* [specimen records preserved at herbarium, Defence Institute for High Altitude Research (DIHAR), Leh, India, voucher specimen No SBTL-2006], were collected from Himalayas. The leaves were shade dried, powdered and soaked in distilled water. The supernatant was collected and lyophilized. The dried powder (yield 0.125 g/g) was coded as SBL-1. To ensure working with standardized extract and also to maintain the quality control, the marker compounds (Quercetin dihydrate, Gallic Acid and Rutin) of SBL-1 were quantified using high performance thin layer chromatography (HPTLC).[25]

Animals and Experimental Procedures

The 8-9 weeks old male, inbred, Swiss albino Strain 'A' mice, weighing 28±2 g, were used after the approval of Animal Experimentation Ethics Committee of the Institute. The animals were maintained under controlled environment at 26±2°C; 12h light/dark cycle and offered standard animal food (Golden feed, Delhi) as well as tap water *ad libitum.* All the animal experiments were conducted according to the guidelines of Committee for Protection and Care of Small Experimental Animals (CPCSEA) and as per the approved protocol.

The animals were divided into four groups. Group I was Untreated Control and was administered vehicle (sterile water) only, group II was total body ^{60}Co-gamma irradiated (10 Gy), group III was Drug alone (administered SBL-1 only, 30 mg/kg b.w.) and group IV was Drug+Radiation (administered SBL-1, 30 mg/kg b.w. and after 30min, animals were total body ^{60}Co-gamma irradiated, 10 Gy).

Each group had three mice. The drug was dissolved in sterile water, filtered and administered intra-peritoneally (i.p). For total (whole) body irradiation, each mouse was placed in a separate wire mesh container and was given one time exposure to deliver total radiation dose (10 Gy), using ^{60}Co-gamma ray source (GC-220, Atomic Energy of

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Canada Ltd., Canada, dose rate 0.31 rad/sec). Fresh air was continuously circulated to avoid hypoxia. Mice from different groups were dissected on three different days (i.e. day 3, 10 and 15).

Isolation of splenocytes, smear prepration and staining

Mice were euthanized by ether inhalation. The spleens from the mice were excised aseptically, blot dried and weighed separately. The ratio of spleen weight to b.w. was recorded. Immediately after recording the weight, the spleens were transferred to chilled phosphate buffered saline (PBS). The cell-suspension was prepared as per the procedure described (https://www.proimmune.com/ecommerce/pdf_files/PR17.pdf).[26] The excised spleens were crushed using the plunger end of the syringe to release splenocytes and the suspension was layered on 1ml of PBS in a 60mm petri-dish for 10min to allow the cellular debris to settle down. The homogenised cell suspension was carefully centrifuged at 400g for 10min at 4°C. The pellet was suspended in a solution containing 2ml of PBS and 1ml of RBC lysing buffer (0.15 M NH_4Cl, 1 mM $KHCO_3$, 0.1 mM EDTA). The suspension was incubated for 15min at room temperature in dark for removal of RBCs. The cell suspension was centrifuged at 400g for 5min at 4°C. The supernatant was discarded and the pellet was resuspended in 3ml of PBS and centrifuged again at 400g for 5min at 4°C. The supernatant was aspirated and the pellet was finally suspended in 1ml of complete RPMI 1640 medium containing 10% FBS, 0.4g sodium bicarbonate, 5000 U/ml of penicillin, and 5000 µg/ml of streptomycin and maintained on ice. 20µl of this suspension was used for preparing the cells smears on glass slides. The slides were air dried and fixed in absolute methanol for 5min and dried again.

To prepare the Giemsa stain stock solution, 1g of Giemsa powder was dissolved in 50ml of glycerol at 60°C by shaking for 1h and then mixed with 50ml of methanol.

For staining the cells on the slide Giemsa staining solution (Giemsa stock: distilled water: methanol: 7:40:4v/v) was used. The slides were stained for 10min, rinsed twice with distilled water, dried and mounted with DPX.

Cell counting

The DPX mounted slides were observed under Upright compound microscope (model-Axio Cam A1; Zeiss, Germany) high power (100X objective and 10X eye piece magnification), under oil immersion.

Thirty positions of each slide were screened at random and the cells belonging to different populations were counted.

Statistical Evaluation

The data presented is the mean ± standard deviation (s.d.) of atleast three replicates. Student's *t*-test was applied for assessing the significance and $P \leq 0.05$ was considered significant.

RESULTS AND DISCUSSIONS

Ratio of spleen weight/bodyweight

Whole body gamma irradiation (10 Gy) significantly ($P \leq 0.05$) reduced the spleen/b.w. ratio at day 3 and 10 in comparison to the untreated controls. Decrease in spleen/b.w. ratio was expected because the total body exposure causes multiple non-recoverable injuries which could lead to decrease in number of splenocytes. All irradiated animals had died by day 12. Treatment with SBL-1 before irradiation, countered the radiation induced decrease in spleen/b.w. ratio. By day 15, there was no difference in spleen/b.w. ratio between group I and group IV animals (Table 1). This indicated the protective action of SBL-1. The SBL-1 treatment alone did not alter the spleen/b.w. ratio significantly at any of the observation days.

Table 1: Effect of whole body gamma irradiation (10 Gy) and drug treatment on spleen/body weight ratio.

Day	Radiation	Drug	Drug + Radiation
3	1.15±0.07*	3.66±0.2	1.27±0.02*
10	1.64±0.60*	4.22±1.2	1.77±0.06*
15	-dead-	3.87±0.41	3.69±0.68

Data are mean ± s.d. of three repeats of each experimental group.

*$P \leq 0.05$ compared to untreated control. The value of untreated control was 3.09±0.13.

Effects on spleen cell populations count

- **Lymphocytes population count**

Whole body irradiation (10 Gy) significantly ($P \leq 0.05$) depleted the lymphocyte population upto 71.8% at day 3 and 76.7% at day 10 when compared with un-treated control. The lymphocytes are radiosensitive and therefore, the reduction in lymphocyte population in spleen was expected. SBL-1 treatment before whole body irradiation also showed decreased lymphocyte population in comparison to untreated control on day 3, 10 and 15. There was a small insignificant increase on day 15 in comparison to day 3 within this group of animals, suggesting that with time the number of lymphocytes were increasing and this could be an important factor for the survival of animals, which were treated with SBL-1 before lethal irradiation (10 Gy).

NK cells are considered as radio resistant cells among lymphocytes. The spleen NK population did not show a significant change in comparison to untreated control in either of the treated groups on all observation days.

- **Monocytes and macrophages count**

Whole body gamma irradiation (10 Gy) significantly ($P \leq 0.05$) decreased the monocytes count (66.67% at day 3 and 55.59% at day 10) in comparison to untreated control (Figure 1).

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However, SBL-1 treatment before whole body irradiation countered the radiation induced decreases, although the difference was not statistically significant till day 10. At day 15, the surviving animals, which were treated with SBL-1 before total body irradiation, showed atleast 44.44% monocytes in comparison to untreated control, indicating the beneficial effects of SBL-1.

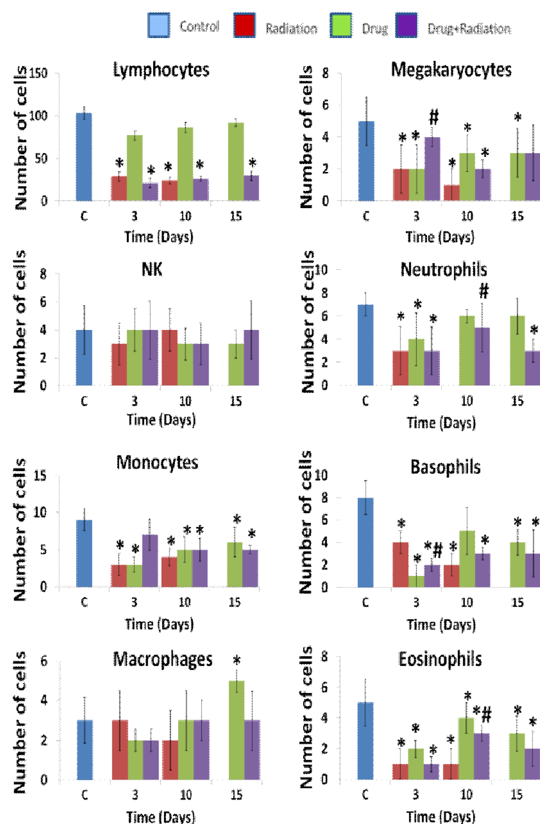

Figure 1: Changes in spleen cell populations with time after whole body gamma irradiation (10 Gy) and drug treatment. Data (mean ± s.d.) were pooled from three repeats of each experimental group. Numbers of mice (n) in each experimental group were 3. *$P \leq 0.05$, compared to untreated control; #$P \leq 0.05$, SBL-1+ radiation compared to radiation control.

Macrophages are radio resistant cells. In this study, no significant change in the macrophages number was observed by any of treatment, in comparison to untreated control.

- **Megakaryocytes count**

Megakaryocytes are located in clusters in the cords of red pulp of the spleen and are responsible for the production of platelets. It is reported that in healthy subjects, the decrease in the platelets in peripheral blood after irradiation, leads to increased production of megakaryocytes in bone marrow.[27] In our study, the whole body exposure to lethal dose (10 Gy) of gamma rays, showed decrease in number of megakaryocytes significantly ($P \leq 0.05$) in a time dependent manner (60% at day 3 and further 80% at day 10) in comparison to untreated control (Figure 1). This in turn indicated the compromised function of bone marrow. However, animals treated with SBL-1 before whole body irradiation showed enhanced megakaryocytes count till day 10 in comparison to irradiation control, but the values were significant ($P \leq 0.05$) only on day 3 (Figure 1). This indicated the radioprotective action of SBL-1. At day 15, the megakaryocytes count was not significantly different than the untreated control, in the animals which were treated with SBL-1 before irradiation.

- **Granulocytes population count**

Whole body gamma irradiation (10 Gy) resulted in a significant ($P \leq 0.05$) reduction in neutrophils (57.14% at day 3; 0% at day 10), eosinophils (80% at day 3; 80% at day 10) and basophils (50% at day 3; 75% at day 10) count in comparison to un-irradiated control, indicating immune-suppressive property of radiation. However, SBL-1 treatment before irradiation countered the radiation-induced decreases in all three cell types, which were significant ($P \leq 0.05$) in case of eosinophils and neutrophils at day 10 (Figure 1). This could be one of the reasons for immune enhancement observed in animals treated with SBL-1 before lethal irradiation (10 Gy).[23] At day 15, the surviving animals, which were treated with SBL-1 before total body irradiation, showed atleast 57.14% neurophils, 60% eosinophils and 62.5% basophils in comparison to untreated control.

Neutrophils are reported to get accumulated at the site of infection by 6h after radiation exposure (4 Gy) and their number increases upto 24h[28] due to innate immune response by chemotaxis (production of chemoattractant molecules at the site of infection). The accumulated neutrophils at the site of infection release large amount of ROS/RNS to combat bacterial infection, a phenomenon commonly known as "respiratory burst".

The animals treated with SBL-1 alone in comparison to untreated control showed insignificant decrease in lymphocytes, NK cells and macrophages on all observation days. In case of macrophages significant ($P \leq 0.05$) increase was observed in comparison to untreated control on day 15. However, in case of monocytes, megakaryocytes, neutrophils, basophils and eosinophils, significant ($P \leq 0.05$) decreases were noticed in animals treated with SBL-1 alone in comparison to untreated control on all observation days. Further studies are planned to confirm these observations by using the cell specific stains.

CONCLUSION

These findings suggested that treatment with SBL-1 before lethal dose of gamma irradiation (10 Gy) could restore the cellularity of spleen in mice by day 15. Since spleen performs multiple important physiological functions, restoration of spleen cellularity by pre-treatment with SBL-1, could importantly contribute to radioprotection in total body lethally irradiated mice. However, the role of SBL-1 needs to be investigated further at molecular level before it can be considered for clinical applications.

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