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Hippophae leaves prevent immunosuppression and inflammation in ^{60}Co-γ-irradiated mice

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Abstract

Hippophae rhamnoides has diverse therapeutic applications in Indian, Chinese and Tibetan medicine. Irradiation (accidental/ therapeutic) causes immunosuppression and inflammation. This study investigated effects of our preparation from leaves of *H. rhamnoides* (code SBL-1), on modification of immunosuppression and inflammation in whole body irradiated (10 Gy) mice. One time treatment with SBL-1 before irradiation prevented the radiation induced (i) decrease in immunoglobulin G, (ii) early release of high mobility group box 1 (HMGB1) protein, (iii) increase in tumour necrosis factor-α, myeloperoxidase activity and lipid peroxidation, (iv) liver haemorrhage, stomach enlargement, spleen shrinkage, intestinal oedema and hair fall, (v) decrease in thiols and ferric reducing ability of plasma. HMGB1, a known therapeutic target, has roles in inflammatory diseases, tissue repair and immunomodulation. This study suggested that SBL-1 countered radiation pathologies by modifying HMGB1 regulated inflammatory pathway and restoring adaptive immune response.

Keywords: *Hippophae* leaves; radioprotection; HMGB1; anti-inflammation; immunomodulation.

Introduction

With increasing use of ionizing radiation in every walk of life, the threat of unwanted radiation exposure and therefore, the subsequent damage, is ever increasing. Depending upon the radiation dose different pathological symptoms appear viz., skin inflammation, decrease in blood cell count, nausea, vomiting, memory loss, immunosuppression and damage to

internal organs. Because of severe immunosuppression, the irradiated tissues become highly susceptible to attack by the pathogens. Irradiation, therefore, causes both infectious and sterile injuries to the internal organs. Development of an agent to counter radiation pathologies is the need of hour for preventing injuries to normal tissues due to radiation exposure that may happen during accidents, therapy and/or occupational exposure.

Increasing number of studies are reporting that High mobility group box 1 (HMGB1) protein is an important therapeutic target (Hanna et al., 2010). HMGB1 has multiple roles in inflammatory diseases as well as in tissue repair. It activates the innate immune response, cell proliferation and regeneration of wounded tissues (Ulloa and Messmer, 2006). HMGB1 gets activated in sterile as well as infectious inflammation/injury (Yang et al*.,* 2010). Intranuclearly localized HMGB1 protein participates in DNA replication, chromatin assembly as well as disassembly; transcription and DNA repair (Bianchi and Agresti, 2005). Extracellularly HMGB1 protein helps in wound healing (Degryse *et al.,* 2001), myocardial regeneration, migration of mesoangioblast cells (stem cells) during tissue damage (Ulloa and Messmer, 2006) and immunomodulation by augmenting maturation of dendritic cells (Dumitriu et al., 2005). Figure 1 schematically reviews different roles of HMGB1. Anti-HMGB1 antibodies conferred therapeutic advantage to mice having tissue injury with lethal sepsis, endotoxemia, cerebral ischemia, ischemia reperfusion injury in liver and heart (Yang and Tracey, 2010). HMGB1 levels were modified by oxidative stress induced by H_2O_2 (Tang et al., 2007) and during radiotherapy of tumours in combination with hyperthermia (Schildkopf et al., 2010).

Hippophae rhamnoides L. (family *Elaegnaceae*) common name Seabuckthorn, is a hardy, deciduous, dioecious shrub which attains 2-4 m height in natural habitat. It is also cultivated at places viz., Western Himalayas, China, Mongolia and Russia. It is a drought resistant plant and can withstand temperatures from -43 °C to 40 °C. *Hippophae rhamnoides* has been used in Indian, Chinese and Tibetan medicinal system for treatment of diverse disorders viz., gastric ailments, circulatory disorders, ischemic heart disease, hepatic injuries, neoplasia (Zeb, 2004). More recently *H. rhamnoides* has been known to have therapeutic role in arthritis (Ganju et al*.,* 2005), wound healing (Upadhyaya et al*.,* 2009), hypoxia (Purushothman et al*.,* 2009) and liver cirrhosis (Gao et al*.*, 2003).

We were the first one to report the protective effect of *H. rhamnoides* leaves (code name SBL-1, under patenting) from lethality caused by whole body ^{60}Co-γ-irradiation (10 Gy). One time intraperitoneal administration of SBL-1 before lethal doses of ^{60}Co-γ-irradiation (10 Gy) rendered significant survival benefit (>90%) in comparison to zero survival in non-SBL-1 treated and irradiated (10 Gy) mice population (Bala et al., 2009). It may be noted that search for a radioprotective agent is a global challenge and till date no radioprotective agent is approved for human use (Weiss and Landauer, 2009). The present study was planned to investigate the effects of SBL-1 on radiation induced inflammation and immune suppression at the radioprotective concentration. HMGB1, since, is a regulatory molecule playing a key role in inflammation as well as immunity and is being increasingly recognized as a therapeutic target, we investigated the modifying effects of SBL-1 on kinetics of HMGB1 release as well as on release of associated cytokines in mice irradiated with lethal doses of ^{60}Co-γ- rays.

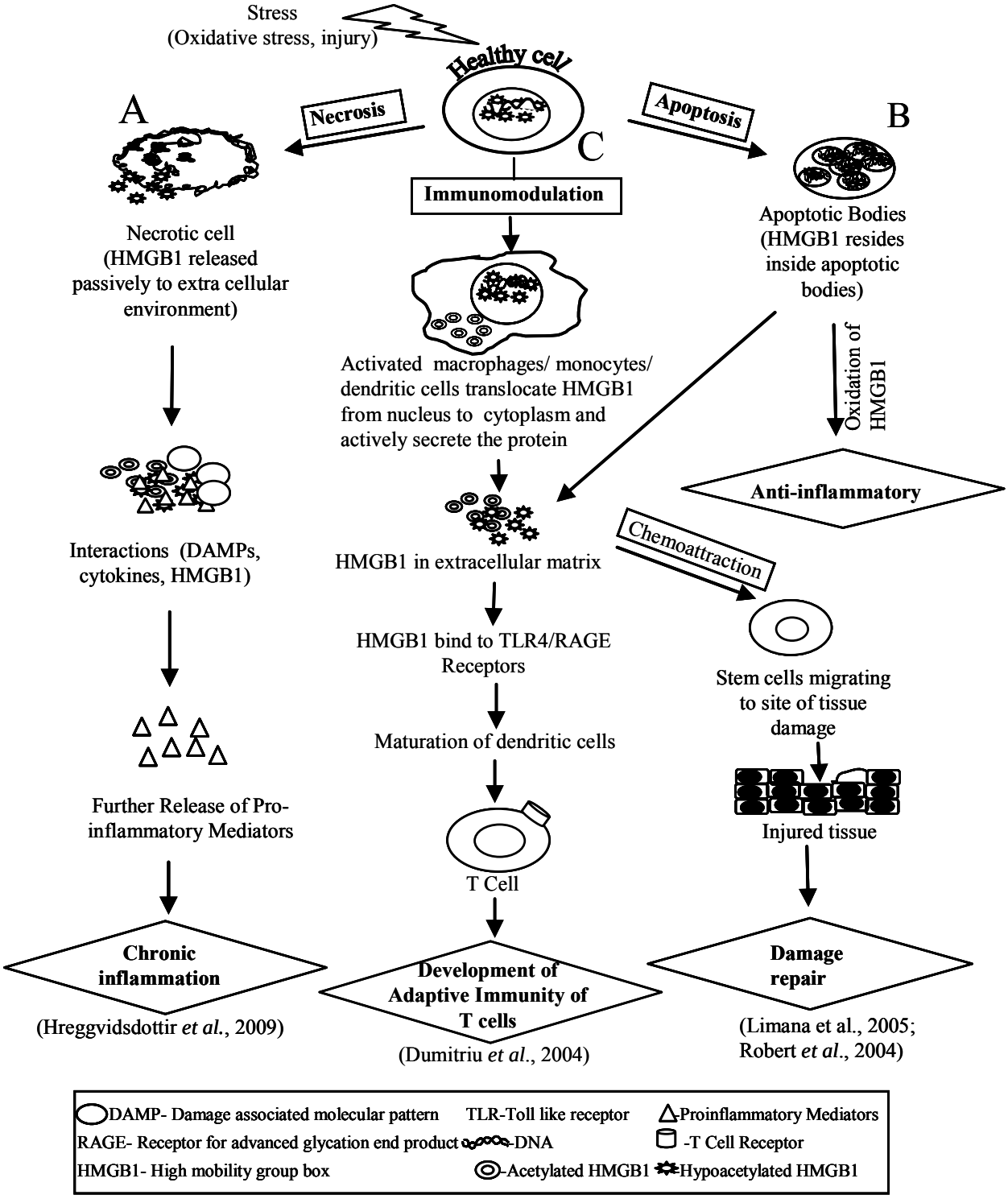

Figure 1. Schematic diagram showing different functions of HMGB1; Pathway A is activated in case of high oxidative stress leading to excessive cellular damage (necrosis). Pathway B is activated when cells subjected to oxidative stress undergo DNA damage which is beyond repair by the inherent reparative enzymes and promote apoptosis. This pathway does not lead to inflammation. Pathway C is initiated when oxidative stress is sensed by cells and the innate immune response is activated without aggravating inflammation.

Materials & Methods

Plant extract and its characterization

Preparation of plant extract and its characterization was described earlier (Bala et al., 2009). Briefly, 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], were collected from Himalayas, shade dried, powdered, soaked in distilled water and supernatant was lyophilized. The dried powder (yield 0.125 g/ g) was coded as SBL-1 and was standardized with reference to quercetin content (Bala et al., 2009). The SBL-1 contained 0.23 $\pm$ 0.002 g polyphenols equivalents of gallic acid, 0.093 $\pm$ 0.008 g flavonoides equivalents of quercetin, and 0.32 $\pm$ 0.006 g tannins equivalents of tannic acid (Tiwari et al., 2009).

Experimental animals

Adult male Swiss albino Strain 'A' mice, 8-10 weeks old, weighing 28 $\pm$ 2 g from an inbred colony were used. The animals were maintained under controlled environment at 25 ± 1 °C and 12 h light/dark cycle, fed standard animal food (Golden feed, Delhi) and were offered tap water *ad libitum.* The mice were sanctioned and issued after the approval of Animal Experimentation Ethics Committee of the Institute. The experiments were conducted in accordance with regulations specified by the Institutional Animal Ethics Committee and conformed to the National guidelines on the care and use of laboratory animals, India.

Experimental procedure

The animals were divided into four groups. Group I- untreated control (treated with vehicle only), group II- whole body ^{60}Co-γ-irradiated (10 Gy), group III- treated with 30 mg/kg body weight SBL-1 (drug) only and group IV- treated with 30 mg/kg body weight drug prior to irradiation (10 Gy). Each group had three mice and each experiment was repeated three times. For all drug treatments, the drug was dissolved in sterile water, filtered and administered intraperitoneally (i.p). For whole body irradiation, each mouse was placed in a separate wire mesh container and given one time exposure to deliver 10 Gy radiation dose using ^{60}Co-γ-ray source (GC-220, Atomic Energy of Canada Ltd., Canada, dose rate of 0.31 rad/sec). Fresh air was continuously circulated to avoid hypoxia.

Mouse necropsy, preparation of liver homogenate, isolation of serum and plasma

Mice were observed daily for visual signs of inflammation. At appropriate time intervals, the animals were sacrificed humanly. Internal organs were observed visually and signs of inflammation viz., oedema, change in size, colour and swelling were recorded digitally using white light camera. Liver was excised, weighed and 10% homogenate was prepared in ice cold phosphate buffer saline (pH 7.2) using homogenizer (Remi motors, Vasai, India). Blood was drawn from anesthetized animals, serum and plasma was collected as per standard procedures.

Detection of total immunoglubulin G (IgG) and cytokines level

Direct enzyme linked immunosorbant assay (ELISA) was used to detect total IgG levels, in mouse serum. The instructions provided by the manufacturer were used to determine the levels of tumour necrosis factor-α (TNF-α, BD Biosciences, USA), Interleukin-10 (IL-10, BD Biosciences, USA) and HMGB1 (IBL-Hamburg, Germany). Absorbance was taken using spectrophotometer (Bio-Tek instrument, USA).

Biochemical Assays

Myeloperoxidase (MPO) activity was determined in serum by the method of Hillegass et al (1990). Ferric reducing ability of plasma (FRAP) was determined by the method of Benzie and Strain (1996). Total thiols in liver were determined by the method reported by Sedlak and Lindsay (1968). Lipid peroxidation (LPx) in liver was determined by the method of Ohkhawa et al (1979). Lpx was measured in terms of malonaldehyde (MDA) formation/mg protein. Proteins were quantified using Bradford assay (Bradford, 1976). All chemicals used for biochemical assays were of analytical grade, purity 99%, procured from sigma.

Statistical analysis

The results were expressed as mean $\pm$ standard deviation (S.D.) of all experimental repeats. Data was subjected to Student's *t*-test and value of $p \leq 0.05$ was considered significant.

Results

Visual examination and necropsy

On day 5, in comparison to untreated control (group I), all animals of group II showed swelling and hair fall at snout region (Figure 2 i) indicating radiation induced inflammation. Necropsies showed that on day 5, the stomachs of group II mice were enlarged (Figure 2 ii), spleen size decreased (Figure 2 iii), pale colour fluid accumulated in intestine (Figure 2 iv) and colour of liver changed to dark red, showing sign of haemorrhage (Figure 2 v). No such pathophysiological symptoms were observed in group III (treated with drug alone) and group IV (treated with drug prior to irradiation) animals.

Immunological studies

In group II animals 1.5 fold increase ($p \leq 0.05$) in HMGB1 level was observed at 2 h, which increased maximum up to 3.1 fold ($p \leq 0.01$) at 8 h (Figure 3a). At 16 h the HMGB1 levels were comparable to group I. In group III animals the HMGB1 levels were not significantly different from untreated control. Group IV animals showed no change in HMGB1 up to 4 h. However, 3.9 folds increase was observed at 8 h and the maximum increase was at 16 h (4.2 fold, $p \leq 0.01$, Figure 3a) in comparison to group I animals.

Increase in TNF-α level was observed from 2 h till 48 h in group II, when compared with the group I animals (Figure 3b). In group III there was no significant difference in comparison to group I. In group IV animals significant increase in TNF-α level was observed at 4 h and 8 h (Figure 3b) when compared with group I.

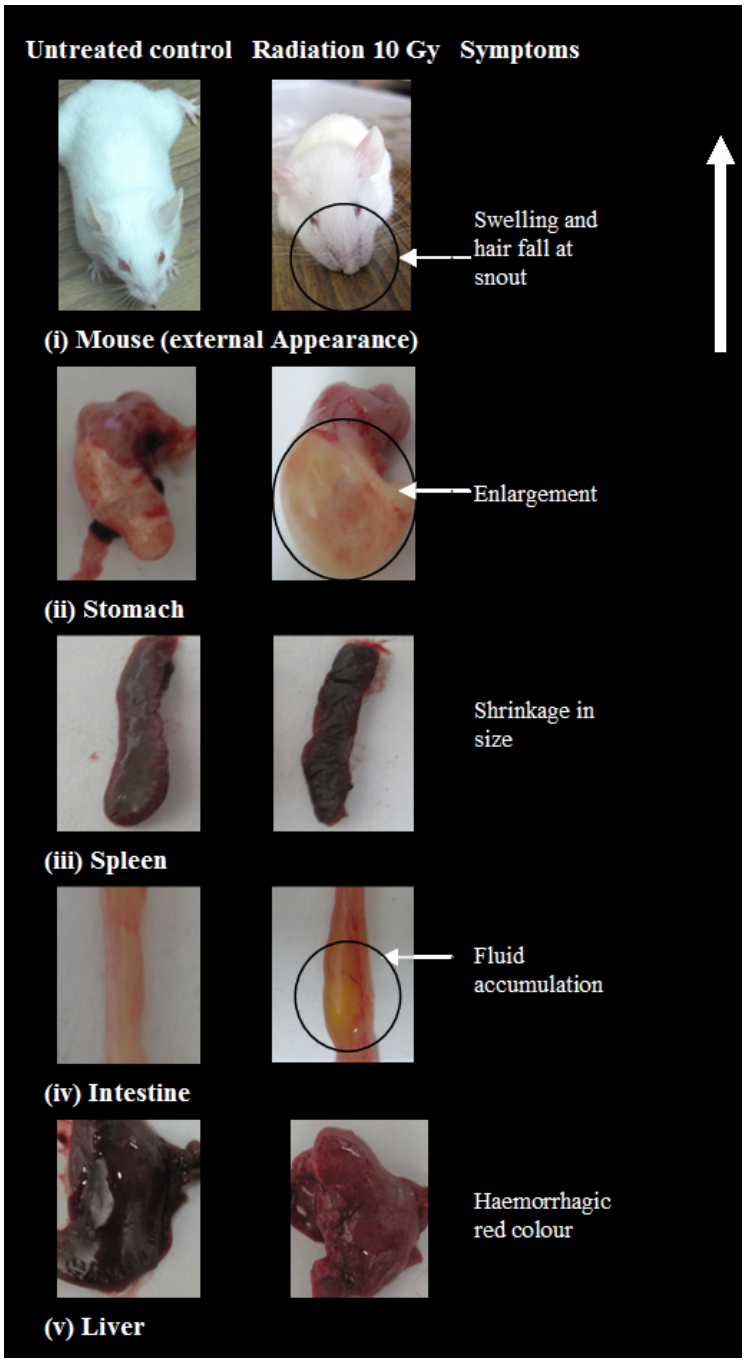

Figure 2. Comparison of untreated control with whole body irradiated (10 Gy) animals after day 5. The animals were necropsied, tissue dissected out and photographed immediately. The animals treated with SBL-1 before irradiation did not show the changes seen in irradiated animals.

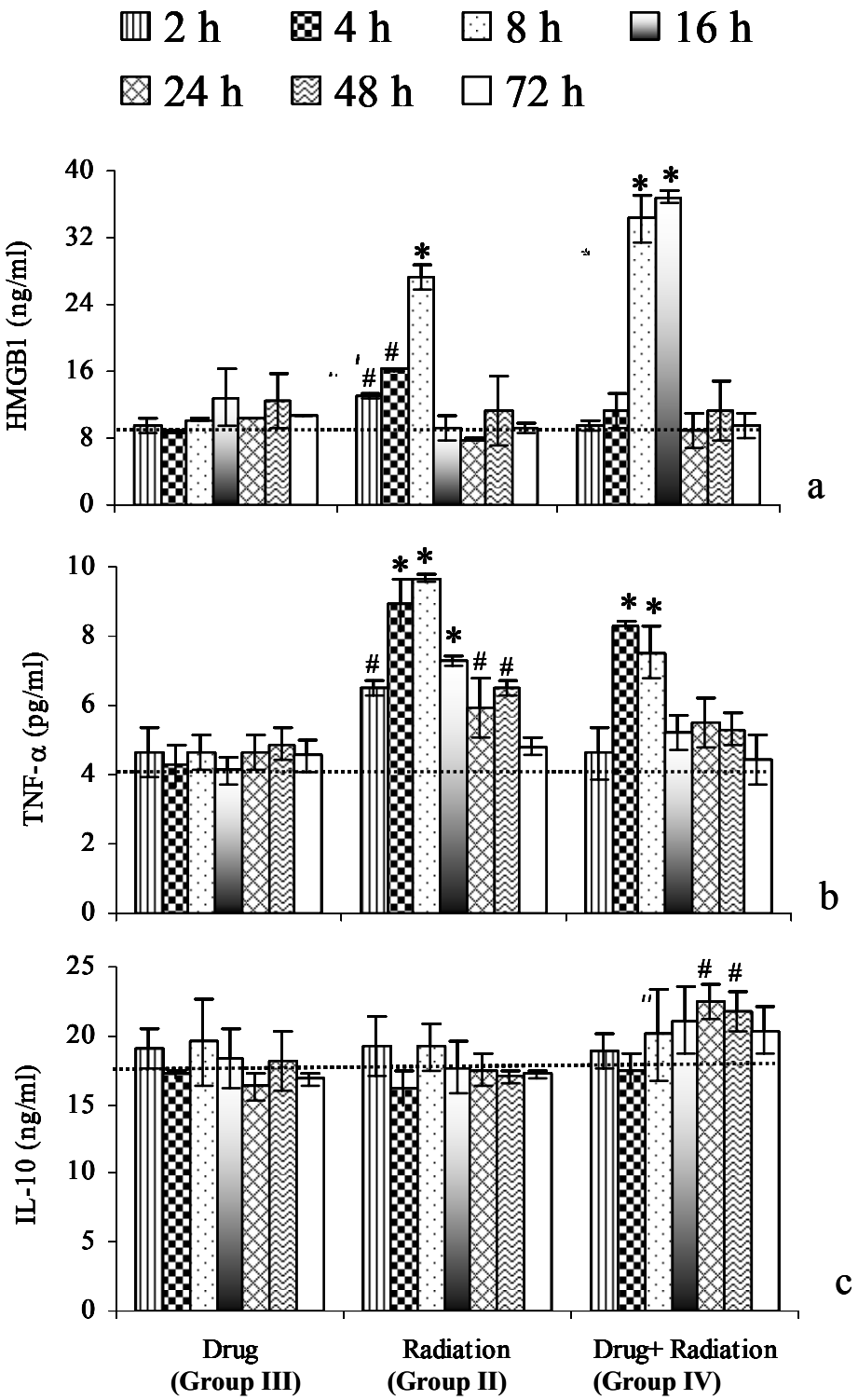

Figure 3. Radiation induced changes and their modification by SBL-1 on levels of (a) high mobility group box 1 (HMGB1), (b) tumour necrosis factor-α (TNF-α), (c) interleukin- 10 (IL-10). Dashed line represents untreated control levels. The data were represented as significant change with reference to untreated control at $p \leq 0.05$ (#) and at $p \leq 0.01$ (*).

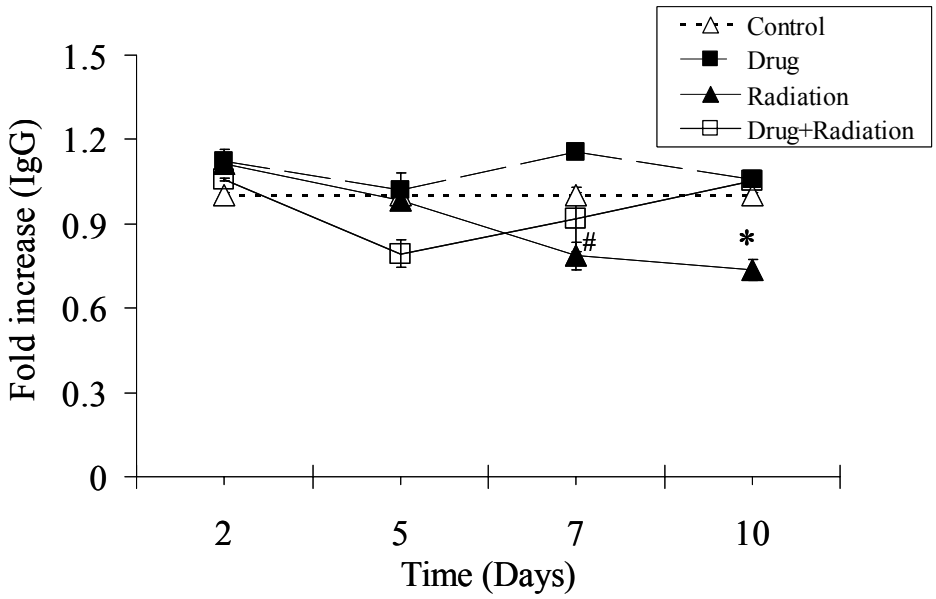

Figure 4. Radiation induced changes in immunoglobulin G (IgG) level and its modification by SBL-1. The data were represented as significant change with reference to untreated control at $p \leq 0.05$ (#) and at $p \leq 0.01$ (*).

The levels of IL-10 in group II and group III did not observably change in comparison to group I. Significant increase (1.4 fold) was observed in group IV animals from 24 h to 48 h only (Figure 3c) when compared with group I. In comparison to group I, the group II animals showed significant decrease ($p \leq 0.05$) in IgG level on day 7 and day 10 (Figure 4). In group III and group IV animals IgG levels were comparable to untreated controls.

Biochemical studies

In comparison to group I animals, the group II animals showed sharp increase in MPO activity at 48 h (1.8 fold, $p \leq 0.01$), which was maintained till day 5 (Figure 5a). In group III animals MPO level increased to 1.5 fold at 24 h only and thereafter, it became comparable to group I. In group IV animals a sharp increase (2.75 folds) in MPO level was observed at 4 h only, which thereafter became comparable to group I (Figure 5a).

In comparison to group I animals, the group II animals showed decrease in FRAP at day 5 and day 10 (Figure 5b). Treatment with drug alone (group III) enhanced the FRAP up to 48 h; maximum increase was 2.9 fold in comparison to group I which was at 16 h after drug treatment. Thereafter, the values were comparable to group I animals. In group IV animals FRAP was significantly more in comparison to group I animals at 8, 16, 24 and 48 h (Figure 5b).

Total thiol content in group II animals decreased significantly in comparison to group I at 2 h, 4 h, day 5 and day 10 (Figure 5c). Total thiol content in group III animals was found 2.2 fold ($p \leq 0.01$) high at 8 h and was significantly high till day 5. In group IV animals significant increase in the thiol content was observed at 16 h onwards till 72 h, when

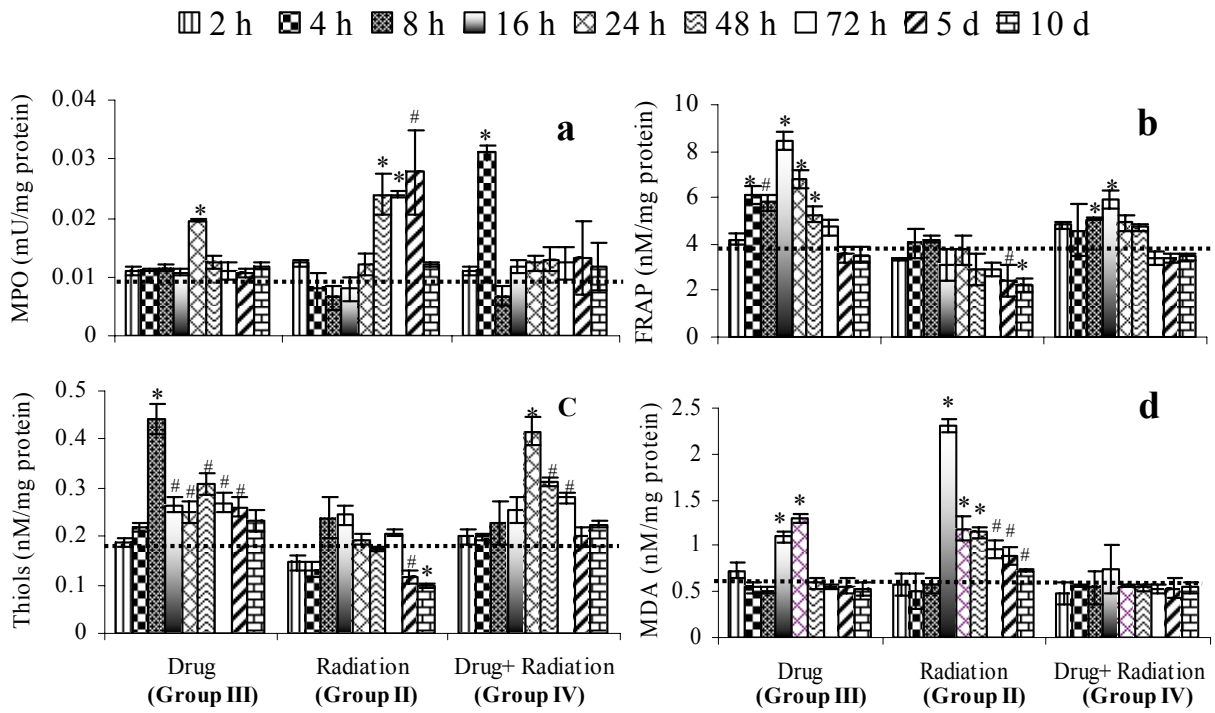

Figure 5. Radiation induced changes and their modification by SBL-1 on (a) myeloperoxidase (MPO) activity in serum, (b) ferric reducing ability of plasma (FRAP), (c) thiol content in liver, (d) lipid peroxidation in terms of malonaldehyde (MDA) levels in liver. Dashed line represents untreated control levels. The data were represented as significant change with reference to untreated control at $p \leq 0.05$ (#) and at $p \leq 0.01$ (*).

compared with the group I animals. In comparison to group II animals the thiol content of group IV animals was higher from 24 h onwards (Figure 5c).

In comparison to group I animals, in group II animals MDA level increased at 16 h (4.6 fold, Figure 5d) and values were significantly high till day 10 (Figure 5d). In group III animals, there was 2 to 3 fold increase in MDA level between 16 to 24 h only. In group IV animals the MDA levels were comparable to group I animals; and were significantly lower than group II at 16, 24, 48 and 72 h (Figure 5d).

Discussion

Irradiation with ionizing radiation (^{60}Co-γ-rays) causes inflammation and immunosuppression in dose and time dependent manner and damages vital biomolecules primarily by producing huge flux of ROS and RNS. Development of prophylactic agents to prevent radiation induced inflammation and immunosuppression are of interest to populations exposed to radiation during accidents, occupation, radiodiagnosis and/or radiotherapy. In this study we evaluated a herbal preparation developed from *H. rhamnoides* (code name SBL-1, under patenting) for its efficacy to prevent radiation induced inflammation and immunosuppression at the radioprotective concentration reported earlier (Bala et al., 2009).

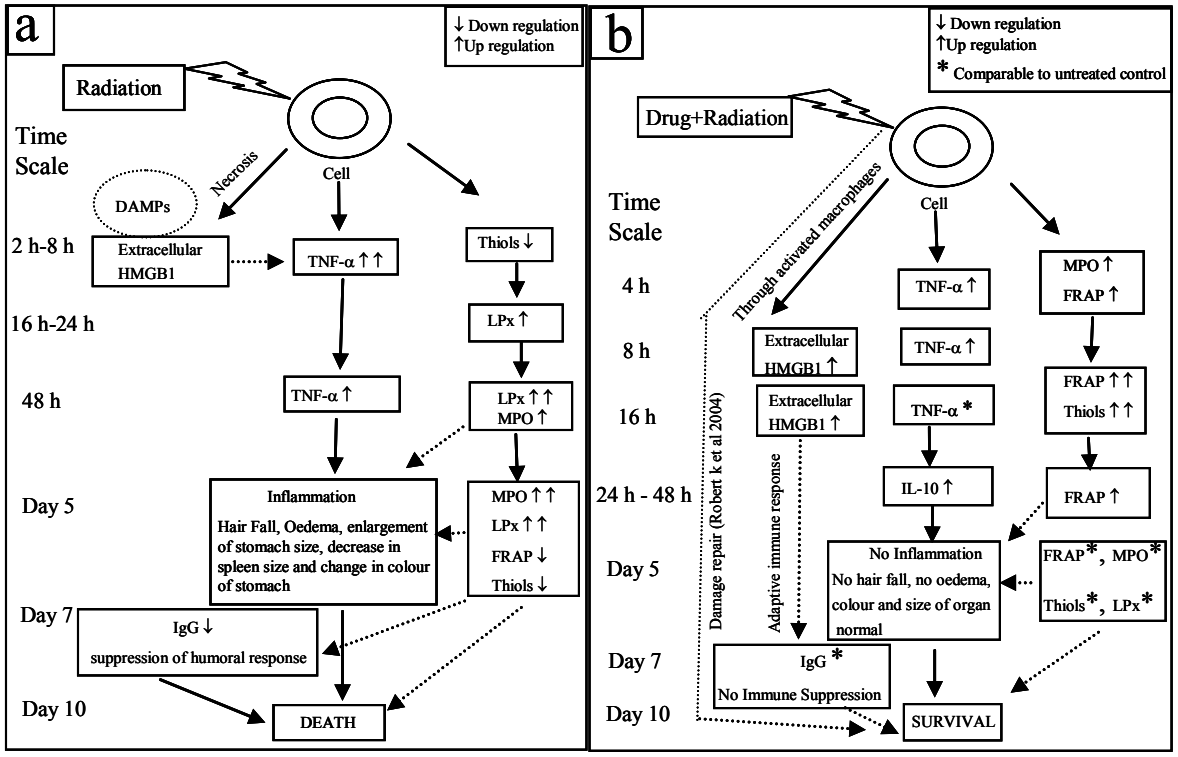

Figure 6. Schematic diagram showing (a) effect of radiation on release of HMGB1 protein, associated cytokines, inflammation and oxidative stress. (b) The modulatory effects of SBL-1 on radiation induced release of HMGB1 protein, associated cytokines, inflammation and oxidative stress. Abbreviation used: High mobility group box 1 (HMGB1), Tumour necrosis factor-α (TNF-α), Interleukin-10 (IL-10), Immunoglobulin G (IgG), Myeloperoxidase (MPO), Ferric reducing ability of plasma (FRAP), Lipid peroxidation (LPx).

The immunoprotective and anti-inflammatory role of SBL-1 was convincingly demonstrated because one time intraperitoneal administration of SBL-1, 30 minutes prior to irradiation prevented the occurrence of radiation induced pathophysiological symptoms viz., stomach enlargement, spleen shrinkage, accumulation of intestinal fluid and liver haemorrhage (Figure 2). Administeration of SBL-1 prior to irradiation delayed the release of HMGB1 protein in extracellular matrix (Figure 3a) suggesting the immunomodulatory effect of SBL-1 through the HMGB1 regulated pathway. SBL-1 treatment before irradiation promoted the increase in IL-10 (24-48 h), normalized levels of TNF-α (16 to 72 h, Figure 3b) as well as of IgG (day 7, Figure 4), confirming the anti-inflammatory and immunoprotective effects of SBL-1 at the molecular level. The SBL-1 being rich in polyphenols, flavonoids and tannins, was shown to have high antioxidant properties *in vitro* (Tiwari et al., 2009; Saini et al., 2010). *In vivo,* SBL-1 treatment prior to irradiation normalized the oxidative stress at day 10 (Bala et al., 2009). Early increase in FRAP (4-48 h, Figure 5b) and total thiols (from 8 h till day 5, Figure 5c), in group III animals, reconfirmed the *in vivo* antioxidant property of our drug within hours of administration. It is proposed that the presence of SBL-1 in the system before irradiation (group IV animals), restricted the radiation induced damage during initial hours by scavenging radiation induced free radicals, ROS, RNS due to its antioxidant properties. This caused removal of damaged cells via apoptotic pathway in contrast to necrotic pathway in group II animals where damage was expected to be massive. The

apoptotic cells are known to activate macrophages/ dendritic cells following pathway B and/or C summarized in Figure 1 where inflammation is not aggravated. The resultant effect was normalization of TNF-α (Figure 3b) and delayed extracellular release of HMGB1 in comparison to group II (Figure 3a). Increase in IL-10 after 24 h (Figure 3c) in group IV animals further supported the anti-inflammatory effect of SBL-1. These events have been summarized in Figure 6.

Short term increase in levels of TNF-α (Figure 3b) and MPO (Figure 5a) immediately after irradiation in group IV animals indicated immediate early pro-oxidant effects. These may have occurred prior to the attainment of desired levels of antioxidants and other bioactive molecules of the drug in the target tissue. Different herbal drugs are known to have different biodistribution kinetics (Grimm et al., 2006). In the later time periods, increase in FRAP (8-48 h, Figure 5b) as well as level of thiols (16-72 h, Figure 5c) and normalization of TNF-α (16-72 h, Figure 3b) and MPO (16h- day 10, Figure 5a) indicated the protective effect of our drug with the passage of time. The pro-oxidant effects of our drug have been reported earlier and were dependent on drug concentration and treatment time (Saini et al., 2010). Short term increase in MDA and MPO levels (Figure 5d and 5a) in group III animals indicated pro-oxidant effect of SBL-1. These pro-oxidant effects were however, short lived and self recoverable. We have earlier reported the benefits of short lived pro-oxidant effects of our herbal radioprotective drugs (Samanta et al., 2004).

To conclude it may be stated that presence of our drug before irradiation in the body, countered the radiation induced inflammatory as well as immunosuppressive effects. Protective effects of drug were evident from the healthy skin, spleen, liver and intestine; prevention of radiation induced oedema and hair loss in animals. Delayed augmentation of HMGB1 in the extracellular matrix in animals treated with drug prior to irradiation, together with activation of anti-inflammatory pathways (normalization of TNF α, and increase in IL-10 levels), immunostimulation (normalization of IgG level) and stabilization of antioxidant defence (increase in FRAP and thiols) importantly contributed to the radioprotective effects of SBL-1. HMGB1 protein, since, regulates multiple pathways such as tissue repair, recruitment of stem cells and immunomodulation, it is expected that our drug has the potential to regulate multiple mechanistic pathways for countering radiation pathologies. Some of the mechanisms of radioprotection by SBL-1 reported earlier were, stimulation of haemopoietic stem cells (Bala et al., 2009) and promotion of error free post irradiation repair resulting in anti-mutagenic and anti-recombinogenic repair of irradiated cells (Tiwari et al., 2009). More studies in this direction are in progress.

Acknowledgement

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Conflict of interest

The authors declare that there is no conflict of interest.

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