Original Article

Protective effect of sesamol against $^{60}$Co $\gamma$-ray-induced hematopoietic and gastrointestinal injury in C57BL/6 male mice

S. Khan$^{1,2}$, A. Kumar$^1$, J. S. Adhikari$^1$, M. A. Rizvi$^2$ & N. K. Chaudhury$^1$

$^1$Chemical Radioprotactor and Radiation Dosimetry Research Group, Division of Radiation Biosciences, Institute of Nuclear Medicine and Allied Sciences, Delhi, India, and $^2$Genome Biology Laboratory, Department of Biosciences, Faculty of Natural Sciences, Jamia Millia Islamia, New Delhi, India

Abstract

Protection of $\gamma$-ray-induced injury in hematopoietic and gastrointestinal (GI) systems is the rationale behind developing radioprotectors. The objective of this study, therefore, was to investigate the radioprotective efficacy and mechanisms underlying sesamol in amelioration of $\gamma$-ray-induced hematopoietic and GI injury in mice. C57BL/6 male mice were pre-treated with a single dose (100 or 50 mg/kg, 30 min prior) of sesamol through the intraperitoneal route and exposed to LD_{50/30} (7.5 Gy) and sublethal (5 Gy) dose of $\gamma$-radiation. Thirty-day survival against 7.5 Gy was monitored. Sesamol (100 mg/kg) pre-treatment reduced radiation-induced mortality and resulted survival of about 100% against 7.5 Gy of $\gamma$-irradiation. Whole-body irradiation drastically depleted hematopoietic progenitor stem cells in bone marrow, B cells, T cell subpopulations, and splenocyte proliferation in the spleen on day 4, which were significantly protected in sesamol pre-treated mice. This was associated with a decrease of radiation-induced micronuclei (MN) and apoptosis in bone marrow and spleen, respectively. Sesamol pre-treatment inhibited lipid peroxidation, translocation of gut bacteria to spleen, liver, and kidney, and enhanced regeneration of crypt cells in the GI system. In addition, sesamol pre-treatment reduced the radiation-induced pattern of expression of p53 and Bax apoptotic proteins in the bone marrow, spleen, and GI. This reduction in apoptotic proteins was associated with the increased anti-apoptotic-Bcl-x and PCNA proteins. Further, assessment of antioxidant capacity using ABTS and DPPH assays revealed that sesamol treatment alleviated total antioxidant capacity in spleen and GI tissue. In conclusion, the results of the present study suggested that sesamol as a single prophylactic dose protects hematopoietic and GI systems against $\gamma$-radiation-induced injury in mice.

Keywords: sesamol, $\gamma$-radiation, hematopoietic progenitor stem cell, bone marrow cells, spleen, gastro-intestine, p53, PCNA, B cells, CD4/CD8, apoptosis

Introduction

Exposures to ionizing radiation cause oxidative injury to almost all organs depending upon the radiosensitivity of the organs, radiation dose, and dose rate [1,2]. These damages result in multi-organ dysfunction, which can lead to acute radiation syndrome (ARS) and long-term health effects, for example, cancer or pulmonary fibrosis [3,4]. ARS includes hematopoietic (2–6 Gy), gastrointestinal (6–8 Gy), and cerebrovascular (> 8 Gy) sub-syndromes [5]. The hematopoietic and gastrointestinal (GI) sub-syndromes are manifested by enormous loss of hematopoietic progenitor stem cells (HPSCs) in bone marrow and impairment of crypt cell regeneration in the GI tract, respectively. Therefore, strategies for developing prophylactic agents as radioprotectors necessarily require an investigation of hematopoietic and GI injury [5].

The possibility of occurrence of ARS during planned radiation exposure exists in critical operations, military warfare, radiation environment, nuclear reactors, and radiotherapy. At present, no radioprotector is available to prevent radiation-induced injuries to hematopoietic and GI systems. Therefore, there is an urgent necessity to develop radioprotectors for human use. Even though the research in this direction was started about five decades ago and amifostine (WR 2721) had emerged as a radioprotector, due to toxicity in human, this molecule was not approved as radioprotector. US FDA has approved amifostine as a cytoprotective agent for patients undergoing radiotherapy of head and neck cancers [6]. Till date, several thousand different chemical and biological compounds have been investigated in vitro and in vivo [5,7,8]. Because of the complex nature of radiation effects in biological systems, all potential molecules have showed lower efficacy, and therefore the possibility of toxicity at higher doses in humans is one of the impediments for the development of radioprotectors. No prophylactic agents are approved by the FDA (USA) to alleviate ARS in humans [9]. Thus, the search for safe, less toxic, or nontoxic prophylactic agents as radioprotectors is continuing [9,10].

Sesamol (3, 4-methylenedioxyphenol) is a natural dietary antioxidant present in processed sesame oil [11],
known for its role in antiaging, chemoprevention, neuroprotection, and hepatoprotection [12–14]. Sesamol is structurally composed of both phenolic and benzodioxole groups, which are responsible for the antioxidative and radioprotective properties, respectively [15]. Sesamol scavenges free radicals, ROS, and nitrogen species by donating hydrogen atoms and enabling electron transfer to the radical center [15]. In addition, sesamol has been demonstrated to have properties that decrease radiation-induced micronuclei (MN), dicentric frequencies, thio-barbituric acid reactive substances (TBARS) and DNA strand breaks, and increase GSH, SOD, CAT and GPx in a concentration/dose-dependent manner in cultured lymphocytes [16,17]. Further, sesamol pre-treatment increased the survival of lethally irradiated mice by inhibiting radiation-induced DNA strand breaks in lymphocytes and lipid peroxidation, and enhancing the level of antioxidant enzymes (GSH, GST, catalase) [18,19]. In view of the promising results, our research group has investigated in detail the antiradical properties of sesamol, along with other known antioxidants. These studies have revealed strong radical-scavenging properties of sesamol in comparison to other reference antioxidant molecules including melatonin, a potential antioxidant-based radioprotector [20]. Further, studies using plasmid DNA (pBR322) and calf thymus DNA, and the in vitro V79 cell line, showed 20 times higher protection and greater DMF (dose-modifying factor) than melatonin, possibly be due to its strong free radical-scavenging property [21].

Based on our earlier studies on sesamol’s antioxidant properties and in vitro results [20,21], as well as literature reports [16,18,19] on radioprotection, we have selected sesamol for further in vivo investigations. The objective of this study, therefore, was to investigate the radioprotective potential of sesamol in whole-body γ-radiated (5 Gy and 7.5 Gy) C57BL/6 male mice, a recommended animal model for developing radioprotectors [22]. A single dose of sesamol (50–100 mg/kg, 1/10th–1/5th of LD50) was selected based on the estimated LD50 dose for the same strain (unpublished data). Sesamol pre-treatment (100 mg/kg) provided 100% survival against LD50/30 (7.5 Gy) dose of γ-radiation. Thus, subsequent studies have been designed at 100 mg/kg of sesamol against LD50/30 (7.5 Gy) and a sublethal (5 Gy) dose of γ-irradiation, to understand the mechanism underlying observed radioprotection in the most radiosensitive organs—the bone marrow, spleen, and GI system [23,24]. We have performed assays of colony forming units (CFU), micronuclei (MN), and cell cycle in the bone marrow; studied immunophenotyping (CD4, CD8), B cells, and apoptosis in spleen; and performed studies on histology, along with lipid peroxidation, and carried out western blotting of apoptotic and anti-apoptotic proteins in the GI system. In addition, gut bacterial translocation to the spleen, liver, and kidney was measured. The results suggest that sesamol pre-treatment protects from radiation-induced injuries in hematopoietic and GI systems in C57BL/6 mice. These findings can be exploited to develop sesamol-based radioprotectors.

Methods

Chemicals

Sesamol (3, 4-methylenedioxyphenol), soybean oil, DPPH (2,2′-diphenyl-1-picrylhydrazyl), protease inhibitor cocktail, anti-p53, anti-Bax, anti-Bcl-x, HRP-conjugate, Bradford reagent, phenyl methyl sulfonyl fluoride (PMSF), bovine serum albumin (BSA), sodium dodecyl sulfate (SDS), Giemsa, DPX solution, sodium phosphate monobasic, sodium phosphate dibasic, concanavalin A (Con A), 2-mercaptoethanol, and propidium iodide (PI) were purchased from Sigma-Aldrich Chemical Co., St. Louis, MO, USA. Tween-20 & 100, skimmed milk powder, ethylene glycol tetra acetic acid (EGTA), Tris-HCl (Tris-Hydrochloride), trichloroacetic acid, phosphate-buffered saline (PBS), HEPES, and sheep blood agar were procured from HiMedia, Mumbai, India. Trypan blue was procured from Spectrochem, Mumbai, India. ABTS (2, 2′-azinobis (3-ethylbenzothiazoline-6-sulfonate), ethylene diamine tetra acetic acid (EDTA), sodium chloride (NaCl), and ethanol were from Merck, Germany, Pen Strep and fetal bovine serum (FBS) were from GIBCO, Invitrogen, USA. RPMI-1640 was from GIBCO, Life Technologies™, USA. Lysis buffer, annexin-V FITC, anti-CD19, anti-CD4, anti-CD8, and anti-PCNA were from BD Biosciences, San Diego, CA, USA. ECL chemiluminescence reagent was from Amersham Pharmacia Biotech, Piscataway, NJ, USA.

Animals

Male C57BL/6 mice (8–10 weeks old) were issued and randomly divided into different groups one week prior to the study, to allow acclimatization. This mouse model is one of the recommended models for developing countermeasures to radiation [22]. Animals not treated (with drug or radiation) served as sham control. Animals treated with sesamol (100 mg/kg body weight) served as sesamol control (sesamol treatment). Mice treated with radiation alone (5 Gy and 7.5 Gy, at 1 Gy/min, whole-body γ-radiation exposure) served as radiation control. The fourth group consisted of sesamol pre-treated mice that received sesamol (100 mg/kg body weight) intra-peritoneally before 30 min of 5 Gy and 7.5 Gy (1 Gy/min) with whole-body γ-radiation exposure. Animals were distributed, with maximum of six per polypropylene cage containing certified paddy husk as bedding, and provided certified food and acidified water ad libitum throughout the experiment. Animals were housed in a pre-maintained room with a 12-h light/dark cycle at a temperature of 23 ± 2°C and relative humidity of 55 ± 5%. All protocols used in this experiment were approved by the Committee on the Ethics of Animal Experiments of the Institute of Nuclear Medicine and Allied Sciences. The Institutional Ethical Committee number under which this study was performed is INM/IEAC/2012/06. All efforts were made to minimize suffering during sacrifice of the animal through cervical dislocation.
Preparation and administration of sesamol

Sesamol was freshly prepared by mixing in soybean oil. A single prophylactic dose of sesamol (50 or 100 mg/kg body weight) or vehicle (soya bean oil) in a volume of 0.2 ml was administered intraperitoneally using a 26-gauge needle, 30 minutes before of whole-body γ-irradiation.

Gamma-irradiation

Animals were placed in a plastic cage and whole-body γ-irradiation exposure was carried out using a 6°Co teletherapy unit (Bhabatron II, Bangalore, India) at a dose rate of 1 Gy/min, to a total dose of 5 Gy and 7.5 Gy. After irradiation, mice were returned to the animal house and monitored. The dose rate of 6°Co γ-rays source (Bhabatron II) was calibrated using physical dosimetry by the radiation safety officer of our Institute.

Immunomagnetic selection of HPSCs and colony forming unit assay in bone marrow

Animals were sacrificed under aseptic conditions, and both the femurs were dissected out. After removing extra tissue from the femur, bone marrow cells were extruded by cutting the epiphyseal ends and flushing using a 26-gauge needle with 5 mL of recommended media (PBS supplemented with 2% FBS). Single-cell suspensions of bone marrow were prepared by gentle pipetting using a sterile Pasteur-pipette (BD Biosciences, San Diego, CA, USA) and passing through a 100 μm nylon mesh strainer (BD Biosciences, San Diego, CA, USA). The marrow cells were centrifuged twice at 500 x g at 4°C for 5 min, and cell numbers were maintained by a hemocytometer (Neubauer, Marienfeld, Germany) using an inverted microscope (4200, Meiji, Japan).

HPSCs (Sca-1⁺ cells) were isolated from whole bone marrow suspensions using the purple EasySep magnet and Mouse Sca-1 Positive Selection Kit (Stem Cell Technology, Canada), following the manufacturer’s protocol. Sca-1⁺ cells (3.6 x 10³ cells) in a volume of 300 μL of recommended media were mixed (by gentle vortex) with 3 mL of MethoCult™ media (Stem Cell Technology, Canada). Using a Luer lock-fitting syringe and a 16-gauge blunt-end needle (Stem Cell Technology, Canada), 1.1 mL of media was dispensed into a pre-marked 35 mm culture dish (Stem Cell Technology, Canada). A 100 mm Petri dish containing two 35 mm culture dishes with lid and a third uncovered 35 mm culture dish filled with sterile water were placed, and incubated for 7–14 days at 37°C, 5% CO₂, with ≥95% humidity. On day 7, the whole 35 mm Petri dish was scanned in a 60 mm gridded scoring Petri dish (Stem Cell Technology, Canada) under low power (40 x and 100 x), and individual colony types (CFU-E, CFU-GM and CFU-GEMM) were identified using an inverted microscope. For each sample, two 35 mm culture dishes were scored, and three mice were used to generate each data point. The total CFU indicated the sum of CFU-E, CFU-GM, and CFU-GEMM.

Bone marrow micronucleus assay

Animals were sacrificed by cervical dislocation on the 1st, 4th, 7th, 15th and 30th days post irradiation. Both the femurs of the animal were dissected and the bone marrow immediately flushed out with pre-chilled PBS. For the MN assay, the bone marrow cell suspension was processed according to Schmid’s method, with minor modifications [25]. Briefly, after centrifugation at 1000 rpm for 10 min, the supernatant was discarded and the cell pellet was resuspended in 2 to 3 drops of FBS. The cell suspension was smeared on clean dry slides, as well as air-dried, and then fixed with methanol. The slides were stained with 4% Giemsa (1/6) diluted in Sorensen’s phosphate buffer (sodium phosphate monobasic and sodium phosphate dibasic, pH 6.8) for 10–15 min, followed by washing with the same buffer. All slides were coded by an independent person and scored under 100 x objectives with a light microscope (Primo Star, Carl Zeiss, Germany). From each mouse, a minimum of 1000 nucleated cells were scored for MN analysis. The frequency of MN per cell was calculated with respect to total nucleated cells scored. The representative images of MN and apoptotic cells are shown in Figure 3. All slides were decoded after completion of scoring and analysis.

Bone marrow cell cycle analysis

Bone marrow cells of animals sacrificed on the 1st, 4th, 7th, 15th and 30th days post irradiation were fixed with methanol. After overnight fixing, 1 x 10⁶ cells were washed with PBS, and then treated with 200 μg/ml of RNase at 37°C for 30 min. Cells were washed with PBS and stained with PI (50 μg/ml). After 20 min, cell cycle analyses were carried out using a BD FACS Calibur 3CB (BD Biosciences, USA) to measure the different phases of the cell cycle.

Flow cytometric analysis of immunophenotyping and assay for apoptosis in splenocytes

Animals were sacrificed by cervical dislocation on the 4th and 21st days post irradiation. The spleen was removed, cleaned, and morphologically observed. The spleen was then minced using sterile slides in a Petri dish containing pre-chilled PBS. Single-cell suspensions were obtained by filtration with a 100 μm nylon mesh strainer (BD Biosciences, San Diego, CA, USA), and RBCs were lysed using BD FACSTM lysing solution (BD Biosciences, San Diego, CA, USA). The single-cell suspension was used for the analysis of CD19 as a B cell marker and CD4 and CD8 as T cell subpopulation markers, and apoptosis was assayed by flow cytometry (LSR II, BD, USA).

To determine the effect of γ-irradiation on the immune system, CD19, CD4, and CD8 levels were examined. Single-cell suspensions were stained following the manufacture’s protocol (BD Biosciences, San Diego, CA, USA). Briefly, the single-cell suspension (1 x 10⁶ cells/mL) was pelleted and mixed with PhycoErythrin-CD19 (PE-CD19),...
Fluorescein IsoThioCyanate-CD4 (FITC-CD4), and Phycocyanin-CD8 (PE-CD8). After an incubation period of 30 min at 4 °C, the pellets were washed twice with washing buffer. In each sample, $5 \times 10^4$ for CD19 and $1 \times 10^5$ cells for CD4 and CD8 were analyzed by flow cytometry. The Annexin V-FITC apoptosis detection kit was used, following the manufacturer’s instructions (BD Biosciences, San Diego, CA, USA). In brief, single-cell suspensions were analyzed by flow cytometry.

**Splenic lymphocyte proliferation assay**

To assess the effect of sesamol pre-treatment on splenocyte proliferation in irradiated mice, mitogen (Con A)-stimulated proliferating splenocytes were evaluated by trypan blue dye exclusion and carboxyfluorescein succinimidyl ester (CFSE) staining [26]. Briefly, animals were sacrificed on day 4 post irradiation by cervical dislocation. Under aseptic conditions, the spleen was dissected out and minced using a pair of sterile frosted slides in autoclaved pre-chilled PBS supplemented with 10% RPMI-1640 and 2% FBS. Single-cell splenocyte suspensions were obtained by passing through a 100 μm nylon mesh strainer (BD Biosciences, San Diego, CA, USA), and RBCs were lysed using BD FACS™ lysing solution (BD Biosciences, San Diego, CA, USA) for 10 min at room temperature. After centrifugation, splenocytes were washed with pre-chilled PBS and resuspended in complete RPMI-1640 media supplemented with 25 mM HEPES (pH 7.4), 50 mM 2-mercaptoethanol, 100 U/ml Pen Strep (penicillin and streptomycin), and 10% FBS. For direct assessment of splenocyte proliferation, the splenocytes ($5 \times 10^6$ cell/ml) were cultured in the presence of Con A (5 μg/ml) and incubated in a humidified atmospheric condition with 5% CO$_2$ at 37°C for 72 h. The splenocyte proliferation was examined by direct counting of viable cells after trypan blue dye exclusion. Alternatively, for assessment of proliferating splenocytes by CFSE staining, the Con A-stimulated splenocytes ($5 \times 10^6$ cell/ml) as described above, were incubated with 1 μM CFSE. The splenocytes were washed twice with PBS supplemented with 10% RPMI-1640 and the pellet resuspended in PBS. The single-cell suspensions were acquired in a flow cytometer (LSR II, BD, USA), and mean fluorescence intensity (MFI) was assessed using FACS Diva software.

**Histological examination in the GI tract**

To evaluate the radiation-induced oxidative damage and recovery in the GI tract, mice were sacrificed on the 1st, 4th, 7th, 15th and 30th days post irradiation and the jejunum dissected out, cleaned in pre-chilled PBS, and fixed in 10% formalin (v/v) at room temperature. Five micron-thick sections were cut and placed on pre-cleaned slides. The sections were stained with hematoxylin and eosin (H & E), mounted and analyzed using an upright motorized compound microscope with a DIC attached and a digital imaging system (Axio, Imager M2, Zeiss, Germany). Qualitative changes in the jejunum of the GI tract were assessed by visualizing the submucosal crypt and jejunal villi. To assess the quantitative changes in the jejunum of the GI tract, ten circumferences were scored per slide for mean crypt and villi numbers, with three mice per group, and a total of $3 \times 10^5$ circumferences per group were used to generate each data point. Ten villi were measured in each section and ten circumferences per slide for a total of $10 \times 10$ villi per mice, and a total of three mice were used to generate each data point for mean villi length. To avoid bias in analysis, all slides were coded prior to staining by a person not involved in the analysis, and decoded after completion of analysis.

**Analysis of gut bacterial translocation**

To analyze the effect of sesamol pre-treatment on the translocation of gut bacteria, bacterial loads were measured as CFUs by culturing spleen, kidney, and liver homogenates on sheep blood agar plates, as described elsewhere [27]. Briefly, mice were killed by cervical dislocation and their abdomens were soaked with 70% alcohol. The spleen, kidney, and liver were collected aseptically on the 9th, 17th, and 21st days post irradiation, with a sterile scissor for each organ. These organs were immediately ground in broth (5 mL each) and incubated aerobically for 24 h at 37°C. After incubation, the organ homogenates were spread with glass rods onto sheep blood agar plates and were incubated for 16 h at 37°C to detect the presence of bacterial CFUs.

**Assay of thiobarbituric acid reactive substances in GI tract**

To assess the level of thiobarbituric acid reactive substances (TBARS) production in GI tract (jejunum), mice were given whole-body $\gamma$-irradiation (5 Gy and 7.5 Gy), and 3 h post irradiation, the jejunum were dissected out by cervical dislocation. Tissue was homogenized in pre-chilled PBS (10% w/v) using a tissue homogenizer (OMNI TH, USA). TBARS were measured following the standard protocol specified by Ohkawa and coworkers [28] in homogenate. TBARS were represented as nmol per gram of wet tissue weight.

**Extraction of protein and western blot analysis in GI Tract, spleen, and bone marrow cells**

The mouse jejunum, spleen, and both femurs were dissected out 3 h post irradiation (5 Gy and/or 7.5 Gy), and extra fat tissue was cleaned in pre-chilled PBS on ice. The jejunum and spleen were homogenized in cold RIPA buffer (50 mM Tris-HCl, 150 mM NaCl, 0.5% sodium deoxycholate, 0.1% SDS, 1% Tween-100, 5 mM EDTA, 1 mM EGTA, and 1 mM PMSF) containing a protease inhibitor cocktail using a tissue homogenizer. Bone marrow cells
were flushed out in pre-chilled PBS, as per the protocol described in the previous section, and lysed in cold RIPA buffer containing protease inhibitor cocktail. The tissue and cell homogenates were centrifuged at 10,000 RPM for 15 min at 4°C. In the same supernatant, protein was measured by the Bradford method [29] and concentration estimated using the BSA standard curve. An equal amount of proteins was separated using 12% SDS-PAGE followed by transfer onto a nylon membrane (Sigma-Aldrich Co.) for 55 min. The membranes were blocked using TBST buffer (0.2 M Tris-base, 1.5 M NaCl, 0.1% Tween-20) supplemented with a solution containing 5% skimmed milk, for 1 h, on a shaker at room temperature. Membranes were then incubated with primary antibodies (β-actin, p53, Bax, Bcl-x and PCNA) overnight on the shaker at 4°C. After washing twice with TBST buffer, the membranes were incubated with secondary antibody conjugated with peroxidase for 1 h at room temperature, on the shaker. The membranes were again washed twice with TBST buffer and bands were visualized using ECL chemiluminescence reagent. The intensity of each protein band was measured using Gel Doc XR (Bio-Rad, USA).

**Analysis of total antioxidant capacity in the GI tract and spleen**

The total antioxidant capacity in the GI tract and spleen was measured in mice treated with sesamol and compared with that of control mice. The GI tract and spleen were removed and cleaned in pre-chilled PBS on ice. Tissue homogenates were prepared in pre-chilled PBS (10% w/v) with a tissue homogenizer, and centrifuged at 12000 g at 4°C for 15 min. The supernatant was stored at −80°C for further analysis.

ABTS radical scavenging activity of the GI tract and spleen was determined spectrophotometrically (Cary100-Bio, Varian, Australia) using the method described elsewhere [30]. The decrease in absorbance of the ABTS radical at 732 nm was measured using 30 min of reaction kinetics. The ABTS⁺ scavenging capacity of the GI tract and spleen were expressed as TEAC (Trolox Equivalent Antioxidant Capacity)/mg of wet tissue. The amount of Trolox (μmol) corresponds to 1 mg of wet tissue.

The DPPH radical scavenging capacity in the GI tract and spleen was measured following the modified procedure [20]. Briefly, prior to analysis, a 200 μM DPPH stock solution was prepared in methanol. DPPH working solution (60 μM) in a volume of 2 mL was mixed with the biological sample (100 μL) and the decrease in absorbance continuously monitored for 30 min using a spectrophotometer at 515 nm. The DPPH radical scavenging activity is expressed as percentage inhibition per mg of wet tissue, as mentioned below.

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\text{DPPH scavenging effect (\% inhibition)} = \left[\frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}}\right] \times 100
\]

Where \(A_{\text{control}}\) is the initial concentration of the DPPH and \(A_{\text{sample}}\) is absorbance of the remaining concentration of DPPH in the presence of antioxidant.

**Statistical analysis**

The mean values and standard errors or percentage of the present data were analyzed and reported. The percentages of survival among groups were analyzed using the Kaplan–Meier statistical graph. For the survival study, p-values at the end of the 10th, 20th, and 30th days post irradiation were analyzed using Log-rank (Mantel–Cox) and Gehan–Breslow–Wilcoxon statistics. Pairwise comparisons were made between groups (inter and intra) using the Student’s t-test and ANOVA (analysis of variance). Statistically significant differences between groups were considered if the value of \(p\) < 0.05.

**Results**

**Radioprotective efficacy of sesamol against \(^{60}\)Co γ-irradiation in C57BL/6 male mice**

Sesamol or vehicle (soya bean oil) was administered intraperitoneally, 30 min prior to 7.5 Gy of whole-body γ-radiation exposure. Sesamol (100 mg/kg) pre-treatment showed survival of about 100% at LD<sub>50/30</sub> dose of 7.5 Gy, whereas a lower dose of sesamol (50 mg/kg) resulted in 70% survival (Figure 1). The vehicle did not exhibit protection and followed an almost similar pattern of death of mice in the irradiated group (Figure 1). The survival study was analyzed on the 10th, 20th, and 30th days post irradiation, with respective percent survival and p-value (Figure 1). The survival data in Figure 1 showed significant protection by sesamol (100 mg/kg) pre-treatment (\(p < 0.05\)), in comparison to mice treated with 7.5 Gy alone, on 20th and 30th day post irradiation. In the present survival study, the first animal died on day 9 in the group exposed to radiation alone, which was expected because of hematopoietic and GI injury. Whole-body radiation dose of 7.5 Gy is known to cause such injuries [1–4]. Therefore, significant survival by sesamol pre-treatment could be due to overcoming the hematopoietic and GI injury. Thus, we have selected 100 mg/kg of sesamol for further evaluation in radiosensitive organs.

**Effect of sesamol on radiation-induced HPSC depletion in bone marrow**

HPSCs are highly sensitive to radiation-induced cellular injury [5]. Therefore, a CFU assay of femoral HPSCs (CFU-E, CFU-GM and CFU-GEMM) was performed to assess the effect of sesamol on radiation-induced hematopoietic injury in mice irradiated with 5 Gy and 7.5 Gy. Whole-body radiation exposure to 5 Gy depicted significant (\(p < 0.001\)) femoral HPSC depletion and reached a nadir on day 4 in 7.5 Gy-irradiated mice (Figure 2). Interestingly, sesamol pre-treatment significantly expanded recovery of femoral HPSCs in 5 Gy and 7.5 Gy (\(p < 0.5\)) whole-body irradiated mice (Figure 2). The CFU assay confirmed that sesamol pre-treatment expands recovery of femoral HPSCs, thereby contributing to overcoming hematopoietic injury in irradiated mice.
Effect of sesamol on radiation-induced micronuclei in bone marrow

The MN frequency was determined in nucleated bone marrow cells of all experimental animals. The results have shown significant increase in the frequency of MN in 7.5 Gy-irradiated mice, in comparison to control mice ($p < 0.001$), 24 h post irradiation. Pre-treatment with sesamol reduced this elevated frequency in irradiated mice. Most importantly, sesamol (100 mg/kg) did not increase MN frequency in bone marrow cells (Figure 3). Irradiation with 7.5 Gy increased apoptotic frequency in bone marrow cells on the 1st day post irradiation, and the frequency was significantly higher ($p < 0.001$) in comparison to control. However, sesamol pre-treatment significantly ($p < 0.05$) reduced radiation-induced apoptotic cell frequency in irradiated mice (Figure 3). On the 7th and 15th days post irradiation, 7.5 Gy-irradiated mice showed very few cells in microscopic slides; only 20–30 cells without MN were observed. On the 30th day post irradiation, all 7.5 Gy-irradiated mice were dead. Pre-treatment with sesamol reduced the MN frequency near to control on 15th and 30th days post irradiation in irradiated mice. The difference was non-significant ($p > 0.05$) in sesamol pre-treated mice compared with control mice. The plot of MN/cell versus post irradiation days for different groups showed the protective effect of sesamol (Figure 3). Therefore, results suggest that sesamol pre-treatment provided significant protection to bone marrow cells against radiation-induced cellular injury.

Effect of sesamol on radiation-induced depletion in B cell and T cell subpopulations

The indirect staining of mouse splenocytes showed significant recovery ($p < 0.01$) of CD19 (B cell), CD4, and CD8 populations in irradiated mice pre-treated with sesamol on day 4 (Figure 6). As expected, the percentage of CD19 cells was clearly low in mice treated with radiation alone, in comparison to control ($p < 0.001$), while an increase was noted in the sesamol pre-treated mice in comparison to mice treated with radiation alone ($p < 0.001$)
In addition, the percentage of CD4 was significantly (p < 0.001) depleted by γ-irradiation (7.5 Gy), and hence, the ratio of CD4/CD8 decreased on day 4 post irradiation. Sesamol pre-treatment significantly (p < 0.01) increased CD4/CD8 ratio on day 4 in comparison to that in mice treated with radiation alone (Figure 6B). The percentage of CD4 and CD8 further decreased on day 21 in comparison to day 4, after exposure to 7.5 Gy of radiation. In contrast, significantly (p < 0.01) higher numbers of CD4 and CD8 were observed on day 21 in comparison to day 4 in sesamol pre-treated mice, therefore, showing recovery in the CD4/CD8 ratio (Figure 6B). Figure 2. Effects of sesamol on femoral HPSCs in mice exposed to γ-radiation (5 Gy and 7.5 Gy, 1 Gy/min) after the 4th day post-irradiation. Femoral bone marrow cells were collected under aseptic conditions by cutting the epiphyseal ends and using 26-gauge needle in 5 mL of recommended media. Femoral HPSCs (Sca-1+ cells) were isolated using purple EasySep magnet and mouse Sca-1 positive selection kit. Sca-1+ cells were mixed with MethoCult™ media and transferred into 35 mm culture dishes using a Luer lock-fitting syringe and a 16-gauge blunt-end needle. All culture dishes were incubated for 7–14 days at 37°C, 5% CO2, with ≥ 95% humidity in a CO2 incubator. On day 7, a 35 mm culture dish was placed in a 60 mm gridded scoring Petri dish and scanned under low power (40x and 100x) using an inverted microscope. Individual colonies like CFU-E, CFU-GM, and CFU-GEMM were identified in each of the 35 mm culture dishes. Two culture dishes were scored for a single mouse and three mice were used to generate each data point. Total CFU indicated the sum of CFU-E, CFU-GM, and CFU-GEMM. *p < 0.001 vs Control, $p < 0.05 vs 5 Gy, #p < 0.05 vs 7.5 Gy.

Gamma-radiation induced significant apoptosis (p < 0.001) on day 4 as analyzed flow cytometrically by Annexin V-FITC and PI staining in mice treated with radiation alone. Sesamol pre-treatment significantly reduced (p < 0.001) apoptosis on day 4 in 7.5 Gy-irradiated mice. In addition, there was an increase (p < 0.01) in the apoptosis on day 21 in comparison to day 4, after exposure to 7.5 Gy of radiation, which was reversed in sesamol pre-treated mice. The basal levels of apoptosis in control mice and mice treated with sesamol alone were found to be similar (Figure 5E). The results suggest that reduction of apoptosis by sesamol pre-treatment might be associated with the increase of B cell and T cell subpopulations in irradiated mice (Figure 6).
Effect of sesamol on radiation-induced proliferation in splenocytes

In order to determine the splenocyte proliferation in 7.5 Gy-irradiated mice, we evaluated PCNA (a nuclear protein and a co-factor for DNA polymerase δ) in 7.5 Gy-irradiated mice, 3 h post irradiation by western blot. A significant decrease in the level of PCNA protein expression was found in 7.5 Gy-irradiated mice in comparison to control (Figure 7A). Sesamol pre-treated mice expressed higher levels of PCNA protein on 7.5 Gy irradiation (Figure 7A). This result indicated that sesamol pre-treatment enhances the expression pattern of PCNA protein, which plays an important role in the processes of proliferation and repair of splenocytes in irradiated mice.

Alternatively, the effect of sesamol on the lymphopoietic capacity of splenocytes in 7.5 Gy-irradiated mice was assessed on day 4 post irradiation by the trypan...
Figure 5. Effect of sesamol on splenocytes hemorrhage, and apoptosis in mice exposed to γ-radiation (7.5 Gy, 1 Gy/min), at the 4th and 21st days post irradiation. The spleen was observed and single-cell suspensions prepared in pre-chilled PBS. Splenocyte single-cell suspensions were stained with Annexin-V-FITC and propidium iodide and analyzed using flow cytometry. Panels A and B are Control and Sesamol, respectively. Panels C and D are 7.5 Gy and Ses + 7.5 Gy on the 21st post-irradiation day, respectively. Panel E is the comparison of % apoptosis between groups. The arrow indicates the point of hemorrhage. @p < 0.001 vs control group, $p < 0.01$ and $p < 0.001$ vs @ and ¥, respectively. @p < 0.05 vs ¥.

blue dye and CFSE staining methods. By direct enumeration under an inverted microscope (4200, Meiji, Japan), we observed a significant proliferation of Con A-stimulated splenocytes from control and sesamol pre-treated mice, in comparison to 7.5 Gy-irradiated mice (Figure 7B). In addition, MFI of irradiated mice were significantly (p < 0.01) increased in comparison to control mice. However, sesamol pre-treatment significantly (p < 0.01) decreased MFI and reached levels near control, in comparison to irradiated mice (Figure 7C). This reduction in MFI was associated with the proliferating splenocytes that underwent division. Thus, results suggest that sesamol pre-treatment enhances the proliferation of splenocytes, which might have led to recovery in the B cell and T cell subpopulations (Figure 6).

Effect of sesamol on radiation-induced damage in the GI tract

In addition to the hematopoietic system, the GI system is also highly sensitive to ionizing radiation. Radiation-induced GI injury is primarily manifested by the death of epithelial crypt cells. The epithelial crypts of the GI region are the highest proliferating cells and most susceptible to ionizing radiation [31]. Thus, counting of the epithelial crypt cells is considered the gold standard method to measure GI injury. It is believed that GI injury is initiated at supra-lethal doses of radiation exposure, but the several recent investigations have revealed GI injury at a much lower radiation dose, also indicating dependency on the radiation dose. Therefore, in this study at the LD₅₀/₃₀, a dose of 7.5 Gy was selected to assess the prophylactic role of sesamol in ameliorating GI injury. Qualitative analysis showed ruptured villi tips and lower numbers of villi and crypts on day 4 in 7.5 Gy-irradiated mice (Figure 8C, E, G and I). Our observations depicted normal histopathological architecture with slight loss of crypts and villi on day 4 in sesamol pre-treated mice (Figure 8D, F, H and J). Sesamol treatment alone did not result in any alterations in the histological architecture of control mice (Figure 8A–B).

Quantitative analysis in the GI tract includes enumeration of crypts and villi, and measuring the length of the villi. A significant (p < 0.001) decrease in viable crypts, villi numbers, and villi lengths was observed on day 4 in 7.5 Gy-irradiated mice (Figure 8K–L). Sesamol pre-treatment increased (p < 0.001) viable crypts, and the number and length of the villi, between day 4 and day 7 in the mice exposed to 7.5 Gy of whole-body radiation (Figure 8K–M). Interestingly, no changes in these parameters were found in control mice treated with sesamol alone till the 30th day of observations (Figure 8K–M). Thus, sesamol pre-treatment protected GI injury by promoting the regeneration of crypts, leading to increase in villi number and length (Figure 8).

Effect of sesamol on translocation of radiation-induced gut bacteria

The effect of sesamol pre-treatment on intestinal mucosal integrity was analyzed by evaluating the translocation of gut bacteria to spleen, liver, and kidney through bacterial CFUs. After exposure to 7.5 Gy of radiation, only a few bacterial CFUs were found on day 9, and no bacterial CFUs were found on day 17 or 21 in sesamol pre-treated mice (Figure 9). In contrast, a large number of bacterial CFUs were found on days 9, 17, and 21 in 7.5 Gy-irradiated mice (Figure 9). However, bacterial CFUs were found at the maximum on day 17 and then decreased in irradiated mice (Figure 9). The number of bacterial CFUs found were higher in the spleen. Thus, the hierarchy of bacterial CFUs in descending order can be demonstrated as spleen > liver > kidney (Figure 9). This result indicates that sesamol pre-treatment results in relatively normal intestinal mucosal integrity in irradiated mice on post-irradiation days.

Effect of sesamol on radiation-induced TBARS in the GI tract

The TBARS level in the GI tract was measured 3 h post irradiation, and showed significant increase in 5 Gy (p < 0.01) and 7.5 Gy (p < 0.001) γ-irradiated mice. However, sesamol pre-treatment reduced the TBARS level (p < 0.01) in irradiated mice (5 Gy and 7.5 Gy). Treatment with sesamol alone did not cause any changes in the TBARS level of the GI tract (Figure 10). This result suggests that sesamol pre-treatment decreases the TBARS induced by ionizing radiation in normal mice GI tract.
Effect of sesamol on radiation-induced p53 and related apoptotic proteins in the GI tract, spleen, and bone marrow cells

It is well documented that the p53 protein plays a vital role in the regulation of radiation-induced apoptotic signaling pathways [32]. To investigate the possible role of sesamol in the regulation of radiation-induced p53-dependent upstream regulators of apoptotic pathways, we examined the differences in the expression of pro-apoptosis (p53 and Bax) and anti-apoptosis (Bcl-x) proteins by western blot, 3 h post irradiation in the GI tract (Figure 11A), spleen (Figure 11B), and bone marrow cells (Figure 11C).

Treatment with sesamol did not cause any changes in the expression of these proteins (Figure 11A–C). In addition, mice irradiated with 5 Gy and 7.5 Gy showed enhanced expression of p53 ($p < 0.0001$) and Bax ($p < 0.001$), in comparison to control, in a radiation dose-dependent manner. Sesamol pre-treatment lowered p53 ($p < 0.001$) and Bax ($p < 0.001$) protein expression patterns in 5 Gy and 7.5 Gy-irradiated mice (Figure 11A). The level of the anti-apoptotic Bcl-x protein in mice was significantly decreased ($p < 0.01$) following exposure to 5 and 7.5 Gy radiation. Sesamol pre-treatment significantly ($p < 0.01$) increased the Bcl-x protein expression pattern in 5 Gy and 7.5 Gy-treated mice. This increased expression of Bcl-x protein was associated with a relatively decreased Bax/Bcl-x ratio in sesamol pre-treated mice, and the ratio was decreased by approximately 2- and 6-fold in 5 Gy- and 7.5 Gy-irradiated mice, respectively (Figure 11A).

The molecular mechanism of a new drug plays a vital role in the drug approval process and is a necessity as per the United State FDA’s “Animal Efficacy Rule”. Therefore, it is crucial to understand the mechanism of a new drug in more than one organ. To prove this hypothesis, we have also measured the expression pattern of p53, Bax, and Bcl-x in spleen (Figure 11B) and bone marrow cells (Figure 11C) of whole-body 7.5 Gy-irradiated mice. Our findings demonstrated that sesamol pre-treatment also decreased the expression pattern of apoptotic proteins-p53 and Bax in the spleen (Figure 11B) and bone marrow cells (Figure 11C). The decrease of apoptotic proteins was associated with the increase of anti-apoptotic protein-Bcl-x, and accordingly, a decrease of Bax/Bcl-x ratio in spleen and bone marrow cells (Figure 11B and C). Thus, here it is hypothesized that part of the radioprotective effects of sesamol may be due to the inhibition of the expression pattern of p53-mediated apoptotic proteins in the GI tract, spleen, and bone marrow cells of whole-body irradiated mice.

Effect of sesamol on total antioxidant capacity in the GI tract and spleen

Total antioxidant capacity (TAC) was measured in the GI tract and spleen after 30 minutes after sesamol treatment.
through the intraperitoneal route. The TAC was measured using ABTS (Figure 12A and B) and DPPH (Figure 12C and D) radical assays. Sesamol treatment significantly (p < 0.01) enhanced gastrointestinal TAC, in comparison to that of control mice (Figure 12A and C). The calculated values of TEAC/mg of wet tissue in the GI tract were found to be 6.5 and 10.1 for control and sesamol-treated mice, respectively (Figure 12A). The values of percentage inhibition of DPPH radical in the GI tract were 55.5 and 72.2, for control and sesamol-treated mice, respectively (Figure 12C). In addition, sesamol treatment also significantly increased (p < 0.01) the TAC of spleen in comparison to control (Figure 12B and D). The calculated values of TEAC/mg of wet tissue in spleen were 7.0 and 8.2, for control and sesamol-treated mice, respectively (Figure 12B). However, the values for percentage inhibition of DPPH radical in spleen were 22.6 and 38.5, for control and sesamol-treated mice, respectively (Figure 12D). Thus, the results indicated that the increase of TAC could overcome the imbalance between pro-oxidants and anti-oxidants in irradiated mice.

Discussion

Poor efficacy and toxicity are the two major impediments in developing radioprotectors. A large number of compounds, both natural and synthetic, have demonstrated potential through in vitro and in vivo model systems [5,7,8]. Cytotoxicity in different organs and systemic levels limit the further progress of these potential candidates. Most of the research on radioprotectors has been focused on the efficacy and mechanism related to the lethal dose of radiation in small animals. However, exposure to a lethal dose of radiation is less likely in planned radiation exposure scenarios, for example, first-responders of radiation emergency, military operations in radiation zones, and also radiotherapy patients for protection of normal tissue. Therefore, radioprotective efficacy of potential candidates needs to be evaluated at lower radiation doses. In this study, therefore, we have performed radioprotective efficacy and pre-clinical evaluations at a safe dose of sesamol (100 mg/kg) (1/5th of LD50 dose) in C57BL/6, based on the estimated LD50 drug dose in the same strain (unpublished data).

In an earlier study, Parihar et al. reported survival of about 70% at a sesamol dose ranging from 50–100 mg/kg in lethally (9 Gy) whole-body-irradiated Swiss albino mice [18]. In the present study, sesamol pre-treatment using 100 and 50 mg/kg provided 100% and 70% survival in 7.5 Gy (LD50/30) whole-body-irradiated C57BL/6 male mice, respectively (Figure 1). This mice strain is a recommended animal model for developing radioprotectors [22]. On careful observations of the previous study in Swiss
albino mice, sesamol dosage of 50 mg/kg showed higher survival as compared to the next higher dosage of 100 mg/kg. This dose–response variation in survival following exposure to lethal radiation could be due to toxicity caused by sesamol itself in this strain, which was not evaluated. Thus, the observation of 100% survival at 100 mg/kg (1/5th of the LD₅₀ drug dose) was novel, and the overall survivability could be due to strain variability. Parihar et al. evaluated GI injury at a supra-lethal radiation dose of 15 Gy using only qualitative analysis at single time points (72 h), and revealed significant protection by sesamol pre-treatment (50 mg/kg), which was not sufficient to assess the recovery end point. In this study, therefore, we have performed both qualitative and quantitative analyses to assess the radiation-induced GI injury in 7.5 Gy whole-body-irradiated mice and found significant recovery as a function of post irradiation days (Figure 8). Further, Parihar et al. had reported benefits of sesamol in lowering splenic injury by evaluating the splenic CFU and splenic index [18]. Thus, we have made an attempt to measure B cell (Figure 6A) and T cell subpopulations (CD4 and CD8) (Figure 6B) and apoptosis (Figure 5E) in the spleen, which plays an important role in immunomodulation. In addition, we have also measured proliferation of splenocytes in presence of Con A by trypan blue dye exclusion (Figure 7B) and CFSE staining (Figure 7C). These results (Figures 1, 5–8) are complementary to the previous study [18], and further strengthen the radioprotective potential.
of sesamol. Kanimozhi et al. reported significant protection against radiation-induced DNA strand breaks in blood lymphocytes by sesamol pre-treatment (100 mg/kg) in 7.5 Gy-irradiated mice [19]. Radiation-induced injuries in hematopoietic systems are manifested by the loss of HPSCs in bone marrow and T cell subpopulations in spleen, whereas GI injury is manifested by the impairment of crypt cell regeneration. These organs (bone marrow, spleen, and GI) are more sensitive to radiation-induced injury in male C57BL/6 mice, in comparison to those of the C3H/He strain [23,24]. As a first step, we have undertaken qualitative and quantitative investigations, revealing that sesamol pre-treatment provided significant protection to these organs against radiation-induced injury in 5 Gy and 7.5 Gy whole-body γ-irradiated mice (Figures 2–12).

Whole-body ionizing radiation exposure can cause hematopoietic and GI sub-syndromes depending on the radiation type, dose, dose rate, and exposure conditions [33]. The hematopoietic sub-syndrome has been reported as a manifestation of the depletion of spared HPSCs, committed to erythrocytes, granulocyte macrophages, granulocyte erythrocyte macrophage megakaryocyte in bone marrow of whole-body irradiated murine model [5]. In addition, the hematopoietic sub-syndrome is also known to complicate into sepsis, and this is a primary cause of mortality in the early phase in animal models. Several cytokines, including growth factors, have been reported to protect mice from radiation-induced mortality when administered as a prophylactic dose. These cytokines and growth factors offer protection by primarily orchestrating and stimulating the host’s innate immune response against ionizing radiation [34,35]. Therefore, cytokines and growth factors are expected to expand the restoration of HPSCs in bone marrow after radiation exposure, and have been demonstrated in pre-clinical and clinical investigations [34,36]. The growth factors GCSF (granulocyte colony-stimulating factor) and GM-CSF (granulocyte-macrophage colony-stimulating factor) are approved for treatment of acute myelosuppression [37,38]. In the present study, femoral HPSCs were measured through the CFU assay and demonstrated significant radiation dose-dependent depletion in whole-body irradiated mice. Sesamol pre-treatment has demonstrated a potential for restoration of femoral HPSCs committed to erythrocytes, granulocytes, macrophages and megakaryocytes in irradiated mice (5 Gy and 7.5 Gy) (Figure 2). In addition, MN and cell death in bone marrow cells were also observed. Radiation-induced MN and apoptotic cells increased 24 h following radiation, and led to cell death at later time points. This was clearly observed as an increased percentage of cell death and MN frequency (Figures 3–4). Cell cycle analysis depicted about 90% cell death on the 4th day (Figure 4A), and appeared to have contributed to animal death observed during observation period of 30 days post irradiation. Sesamol pre-treatment reduced radiation-induced MN frequency and apoptosis to levels near control on the 15th day post irradiation (Figure 3). Further, sesamol pre-treatment also significantly reduced bone marrow cell death, as seen in the cell cycle analysis.
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recovery of B cell and T cell subpopulations (Figure 6), pre-treatment inhibited apoptosis (Figure 5E) and enhanced 7.5 Gy-irradiated mice (Figure 5C). However, sesamol contributed to the observed hemorrhage in the spleen of and decrease in lymphocyte populations appears to have spleen (Figure 6B). This increase in apoptosis (Figure 5E) irradiation also reduced the maximum numbers of B cell Gy of whole-body radiation (Figure 5E). At the same time, FITC) were detected in the spleen after exposures to 7.5 ent study, higher levels of apoptotic cells (Annexin-V-rhage, and can cause malfunction of the adaptive immune immune response (3). Among the cells that comprise the adaptive immune response, CD4 and CD8 are vital, with functional relevance in cell-mediated immunity, while CD19 is relevant in humoral immunity. Studies have reported apopto-sis mainly in the white pulp region of the spleen due to immodulation. The hematopoietic tissue (spleen) is composed of lymphocytes and their subpopulations having differential rad-Loss of T cell subpopulations (Th and Tc) results in thrombocyto-penia or hemorrhage, and can cause malfunction of the adaptive immune response [3]. In the GI tract, epithelial crypts are the highest pro-liferating cells [30,43], and this makes them the most sus-cceptible to ionizing radiation [31]. Therefore, death of epithelial crypts is manifested as the pathogenesis of the GI sub-syndrome [43]. The GI sub-syndrome is believed to appear at 10 Gy and above in mice, but recent studies have also reported GI injury at lower radiation doses [43]. Apoptosis has been reported in the intestinal crypts (stem cell region) within 3–6 h following exposure to radiation of 1 Gy [31], and increases subsequently with radiation dose [43]. Ionizing radiation-induced free radicals mediate oxidative damages, for example, lipid peroxidation, DNA strand breaks, and subsequent alteration in the anti-apoptotic and pro-apoptotic proteins, manifested as mal-absorption in the GI tract (acute bowel reaction and radiation proctitis) [30,31,44–46]. Ultimately these effects lead to fluid and electrolyte imbalance, bacteremia, endo-toxemia, and subsequent death of epithelial crypts [47]. Therefore, protection of the epithelial crypt cells is of utmost importance for the prevention of radiation-induced pathogenesis of the GI sub-syndrome. A number of compounds have demonstrated potential for protection against radiation injury in GI in animal models. These are nutra-ceuticals (vitamin E and vitamin A) and nutritional com-pounds, methyl xanthenes, interleukin-11, prostanoids (prostaglandins, prostacyclins), and other biological and chemical agents [48]. However, no specific agent is avail-able for protection of the GI system.

Injury in the GI is observed as early as 30 min after radiation exposure. Further, repair and regeneration of epi-
Epithelial crypt cells in the radiation-exposed GI tract may be completed within 3–days [49]. Therefore, in this study, early measurement of radiation-induced GI injury was initiated after 3 h for lipid peroxidation and altered expression patterns of anti-apoptotic and pro-apoptotic proteins in mice irradiated with sub-lethal (5 Gy) and LD50/30 (7.5 Gy) doses. Histopathological changes and gut bacterial translocation were assessed from day 1 and day 9 in 7.5 Gy-irradiated mice, respectively. Our data has shown that sesamol pre-treatment can significantly lower lipid peroxidation in the GI tract of mice irradiated with 5 Gy ($p < 0.001$) and 7.5 Gy ($p < 0.001$) (Figure 10). In addition, more number of surviving crypts and villi as well as increased villi lengths were observed in sesamol pre-treated mice (Figure 8). Further, sesamol treatment increased GI tissue’s TAC (Figure 12), which might have led to an increased scavenging of ROS, and hence decreased ROS-mediated injury in irradiated mice.

Elucidation of the molecular mechanism of a radioprotective drug in more than one organ is crucial and plays a vital role in the process of approval of a new drug. To our knowledge, no study has reported the molecular mechanism of sesamol in the amelioration of γ-ray-induced GI tract, spleen and bone marrow injury in mice. Therefore,
the molecular mechanism underlying sesamol-GI tract, spleen, and bone marrow cytoprotection in irradiated mice was studied. It has been well-established that ionizing radiation induces p53 activation in response to DNA damage, which plays a vital role in the activation and mobilization of several pro/anti-apoptosis markers [50]. However, molecular mechanisms besides Bax induce apoptosis by mobilization of Bax oligomers to mitochondria through p53 activation. The anti-apoptotic marker, Bcl-2, is known to counter Bax action and thereby inhibit apoptosis. Therefore, a balanced pro-apoptotic versus anti-apoptotic protein (Bax/Bcl-2) ratio plays a vital role during cell survival [51]. Our present study of the molecular mechanism in the GI tract, spleen, and bone marrow has indicated that sesamol pre-treatment inhibited radiation-induced expression of p53 and Bax (Figure 11A–C). The inhibition of the pro-apoptotic protein-Bax was associated with the increase of anti-apoptotic protein-Bcl-x resulting in balanced pro-versus anti-apoptotic protein expression in sesamol pre-treated mice (Figure 11A–C).

Ionizing radiation induces translocation of bacteria from the gut to different organs, which plays an important role in the pathogenesis of radiation-induced GI sub-syndromes [52,53]. Extensive bacterial translocation from the gut to the spleen, liver, and kidney can lead to sepsis and other complications [3,54]. In this study we have reported the effects of sesamol pre-treatment on the mucosal integrity of GI system by counting the bacterial CFUs in different organs (spleen, liver, and kidney). Our data (Figure 9) suggest that sesamol pre-treatment inhibits translocation of gut bacteria to spleen, liver, and kidney, and maintains mucosal integrity of the GI tract. In the present survival study, about 50% of mice died between the 9th and the 24th days at the LD50/30 radiation dose of 7.5 Gy, which could be due to increased bacterial growth in the spleen, liver, and kidney. Therefore, sesamol pre-treatment significantly protected loss of intestinal mucosal integrity, and contributed to 100% survival at the LD50/30 radiation dose of 7.5 Gy (Figure 1).

In conclusion, the present study has demonstrated that a single prophylactic dose of sesamol pre-treatment reduced lethality in γ-irradiated mice. Sesamol treatment increased TAC in the hematopoietic (spleen) and GI tissues. Sesamol pre-treatment provided protection in the hematopoietic system by expanding the restoration of HPSCs and lowering MN in bone marrow cells. In addition, sesamol pre-treatment increased the splenic PCNA and lymphoproliferative capacity, which might lead to recovery in B cell and T cell subpopulations in the spleen. Sesamol pre-treatment also protected the GI system by inhibiting radiation-induced lipid peroxidation and gut bacterial translocation, and enhancing crypt cell regeneration and antioxidant capacity. The observed radioprotection in these organs (GI tract, spleen, and bone marrow) might be due to the inhibition of radiation-induced p53 and Bax pro-apoptotic signaling proteins. This decrease of pro-apoptotic proteins has been associated with the increase of the Bcl-xL anti-apoptotic protein. This study suggests that sesamol pre-treatment is an effective prophylactic agent for preventing radiation-induced hematopoietic and GI injury in C57BL/6 mice. Therefore, the present results provide useful information for developing sesamol-based radioprotectors.

Figure 12. Effect of sesamol on total antioxidant capacity in spleen and GI tract. At 30 min of sesamol administration, GI tract and spleen were dissected out, cleaned, and used to prepare homogenates (10% w/v) in pre-chilled PBS. Total antioxidant capacity was measured by ABTS and DPPH radical assays using a spectrophotometer. Panels A and B show the GI tract and spleen using ABTS assay. Whereas, panels C and D show the GI tract and spleen using DPPH assay. @p < 0.01 vs control group. $p < 0.05$ vs control group.
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Declaration of interest

The authors report no declarations of interest. The authors alone are responsible for the content and writing of the paper.

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Supplementary material available online

Supplementary Figure 1 to be found online at http://informahealthcare.com/doi/abs/10.3109/10715762.2015.1071485.