INTRODUCTION
Exposure to ionizing radiations is common in the modern age as they are widely used in research, diagnosis, manufacturing and construction (Brenner et al., 2007). Ionizing radiation is a common modality of treatment of cancer patients. Haematopoiesis maintains blood cell lineages at constant level. Bone marrow provides a favorable microenvironment for hematopoietic stem cells, enabling repopulation, differentiation and migration, and also regulates generation of blood cells (Shen et al., 2010). The extremely proliferative

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property of the HSCs (Ogawa M., 1993) required to maintain homeostasis makes them highly radiosensitive (Chinsoo et al., 1998; Chitteti et al., 2011; Till et al., 1964). A radiation dose of 2-8 Gy may create significant damage to the bone marrow causing the hematopoietic syndrome of the acute radiation syndrome (HS-ARS), characterized by life-threatening lymphocytopenia, neutropenia, and thrombocytopenia, and possible death due to infection and/or bleeding (Anno et al., 1989; Coleman et al., 2001; Simopoulos AP, 2002).

Diet plays a key role in normal functioning and development. \(\omega-3\) (n-3) and \(\omega-6\) (n-6) polyunsaturated fatty acids (PUFAs) are important structural and functional components of cell membrane phospholipids. These form the essential fatty acids, as they cannot be synthesized in the human body and must be obtained from diet (Gebauer et al., 2006). As essential nutrients obtained only through dietary intake, their tissue content in individuals can vary, but may be modified through dietary intervention. The beneficial effects of DHA and AA are observed in humans and animal models of diabetes, obesity, cancer, hypertension, autoimmune disorders, mental health, and cardiovascular diseases, etc. They play an important role in embryonic development, development of vision and neuronal development. The metabolites play key role in cell signaling, and thereby modulate various physiological and pathological processes (Belluzzi et al., 1996; Ismail HM, 2005; Shannon et al., 2007; Simopoulos, 2009). PUFAs also get incorporated in membrane lipid raft, consequently altering the membrane composition (Turk et al., 2013). These lipid rafts have important role in embryonic stem cell self-renewal (Lee et al., 2010). The metabolism of AA/DHA is depicted in the flow chart below (Fig. 1). AA is broken down to either leukotrienes, prostaglandins or eicosatetraenoic acids by lipoxygenases, cyclooxygenases and cytochrome P450, respectively. Similarly, DHA is metabolized through lipoxygenases to resolvins.

The effect of PUFAs on haematopoiesis is complex, since these fatty acids are processed into leukotrienes, eicosanoids and prostaglandins, which independently affect haematopoiesis. Several reports suggest that the PUFAs act on human marrow myelopoiesis and erythropoiesis as evidenced by the growth of committed progenitors (CFU-GM and BFU-E) in
vitro (Dupuis et al., 1997). It has been reported that diet rich in n-3 PUFAs relative to the proportion of n-6 PUFAs, affects myelopoiesis by reducing total myeloid progenitor cell frequency and promotes differentiation of specific progenitor cell types in the bone marrow of mice (Verny et al., 2009). Besides, AA and DHA influences megakaryopoiesis and thrombopoiesis in vitro (Shabrani et al., 2012; Siddqui et al., 2011).

Thus, our hypothesis was 'whether oral feeding of PUFAs in hematopoietically compromised mice, enhances haematopoiesis in mice'. In the present study, we demonstrate that feeding sub-lethally irradiated mice with DHA or AA orally for ten days enhances the bone marrow cell count and increases haematopoiesis.

**MATERIALS AND METHODS**

**Mice**

Protocols used in the animal experimentation were approved by the Institutional Animal Ethics Committee (IAEC). C57BL/6 mice (6–8 weeks old, females) were used for the feeding experiments.

**Nutraceuticals**

Docosahexanoic acid (> 99% Pure) and Arachidonic acid (> 99% Pure) were procured from NuChek Prep (Elysian, USA).
Oral Feeding of Mice With Nutraceuticals
The following protocol was followed: 1) Mice were fed various doses of AA/DHA: 2, 4, 8 and 16 mg. Control mice were fed with PBS (vehicle control). 2) Control and test mice were subjected to dose of 4.5 Gy irradiation using \(^{60}\)Co Gamma Chamber (BRIT, Mumbai, India) and kept on normal diet for ten days. Non-irradiated mice were kept as control. 3) Control mice and test mice were sub-lethally irradiated as described above. Test mice in addition to normal solid feed were fed 8 mg AA/DHA daily through oral feeding gavage in separate sets for ten days. Mice fed with PBS (henceforth will be referred as unfed) were used as control.

Harvesting and Processing of BM and PBL
Mice from all experiments were sacrificed after ten days and their bone marrow mononuclear cells (BM-MNCs) were harvested by flushing tibia and femur bones with 21G syringe. Total nucleated cells (TNCs) were counted manually using hemocytometer after mixing them with Turk’s solution containing crystal violet and acetic acid. They were further subjected to flow cytometry analysis of HSCs like SP and LSK analysis.

Blood was collected from mice that were irradiated and then fed with PBS/AA/DHA and was subjected to hemogram analysis using automated blood cell counter.

Side Population (SP) Analysis
SP analysis was performed as described by Eaker et al. (Eaker et al., 2004). Briefly, \(10^6\) BM MNCs of fed or unfed mice were stained with 5 \(\mu\)g Hoechst 33342(Sigma), with or without 50 \(\mu\)M Verapamil (Sigma Aldrich, St Louis, USA), for 90 min at 37°C. The cells were stained with 50 \(\mu\)M Propidium Iodide (PI) for detecting dead cells. The cells were analyzed on a flow cytometer (FACS ARIA III SORP, Becton Dickinson) using UV laser.

Phenotypic Analysis
LSK analysis was performed as per Uchida et al. (Uchida et al., 1992). Briefly, \(10^6\) BM MNCs were suspended in IMDM containing 20% FBS. The cells were washed and suspended in PBS containing 0.1% BSA and 0.1% sodium azide, and stained with c-Kit CD117-PE-Cy7, CD45.2-PB, lineage marker cocktail (CD3e, CD11b, CD45R/B220,
Ly-76, Ly-6G, and Ly-6C)-APC, Sca-1/Ly-6A/E-PE (BD Bioscience, San Diego, USA), at 4°C for 45 min with frequent mixing. The cells were washed with PBS and fixed in 1% buffered paraformaldehyde. Appropriate isotype controls were used. Fifty thousand events in the lineage negative gate were acquired for each sample (FACS Canto II; BD Bioscience, San Diego, USA). The flow cytometry data was analyzed using FACS Diva™ (BD Bioscience) software. c-Kit and Sca-1 double positive population was gated in lineage negative cells to get LSK population.

Statistical Analysis
Statistical analysis was done using Sigma Plot 11 (Jandel Scientific Corporation, San Rafael, California, USA) software using One Way RM-ANOVA. The mean and standard deviation obtained was plotted for the various assays. The data was considered significant if P < 0.05 (*), P < 0.01 (**) and P < 0.001 (***) Graphs were plotted using the same software.

RESULTS
Optimal Dose of AA/DHA for Haematopoiesis in Mice
To determine optimal dose of AA or DHA for haematopoiesis, mice were fed for ten days with 2, 4, 8 or 16 mg of AA or DHA, respectively. PBS fed (Unfed) mice were kept as sham control. Mice were sacrificed after 10 days of feeding and their bone marrow mononuclear cells (BM-MNCs) were subjected to total nucleated cell (TNC) count, SP and LSK analysis. As shown in the Fig. 2A, BM MNCs of mice fed with 8 mg AA /DHA showed significantly higher number of TNCs as compared to control mice (PBS fed), indicating 8 mg as the optimal dose. Side population cells are known to give prolonged multi lineage haematopoiesis since they harbor long-term repopulating stem cells. Fig. 2B, shows representative FACS profile of fed and unfed mice for SP cells. Specificity of SP phenotype was confirmed by addition of Verapamil known to abolish SP profile. Cumulative data from five mice indicate that oral dose of 8 mg of AA/ DHA was optimal for stimulating side population (Fig. 2C). LSK cells are known to be primitive stem cells. As observed in Fig. 2E, marrow cells of the AA-fed and DHA fed mice showed higher percentage of LSK cells as compared to the controls. Representative FACS profile is depicted in Fig. 2D. Thus the data show that oral dose of 8 mg of AA or DHA enhances haematopoiesis in mice.
Sub-lethal Irradiation Depletes Bone Marrow Cells and Hscs in Mice

To study the effect of irradiation on haematopoiesis, mice were given sub lethal dose (4.5 Gy) of irradiation; healthy, non-irradiated mice were kept as control. Mice were kept untreated for 10 days and sacrificed after 10 days. The bone marrow cells were harvested and analyzed for TNC count, SP cells and LSK cells. Fig. 3A shows that sub lethal irradiation significantly depleted total nucleated cells in mice. Flow cytometry profile (Fig. 3B) and cumulative statistical data in Fig. 3C shows more than two fold reduction in side population cells of irradiated mice. Sub lethal dose of gamma irradiation causes...
Irradiation decreased primitive stem cells as observed by decreased percent LSK (Fig. 3E). Representative flow cytometry profile is depicted in Fig. 3D.

Feeding AA or DHA to Sub-lethally Irradiated Mice Restores Haematopoiesis

Sub-lethally irradiated mice were fed with AA or DHA for ten days. Mice were sacrificed and bone marrow (BM) cells
and peripheral blood (PBL) cells were harvested. Total nucleated cells were increased in BM MNCs of AA or DHA fed mice, as compared to control mice (Fig. 4A). AA/DHA stimulated long term repopulating cells. As shown in Fig. 4B, increased percentage of SP cells was observed in AA (2.1%) and DHA fed mice (1.6%). Cumulative data clearly indicates that AA and DHA caused significant enhancement in the percentage SP in the bone marrow (Fig. 4C). AA and DHA stimulated primitive stem cells. Fig. 4D shows increased percentage of LSK cells in AA fed (1.9%) and DHA fed (2.3%) mice. Significantly increased number of LSK cells were observed in bone marrow of fed mice (Fig. 4E).

Peripheral blood cells of unfed and
fed mice of this set of experiment were subjected to hemogram analysis. It was observed that feeding of AA and DHA resulted in increased RBC (Fig. 5A) and platelet count (Fig. 5B) in mice. AA and DHA significantly increased leucocytes especially lymphocytes in the peripheral blood of mice (Fig. 5C).

Thus, the data suggests that oral administration of AA or DHA to normal healthy mice stimulates the haematopoiesis. When sub lethally irradiated mice were fed with DHA/AA, it stimulated their long-term repopulating cells, primitive HSCs and promoted enhancement of erythropoiesis and thrombopoiesis.

DISCUSSION
In the present study, we have made a systematic attempt to examine the effect of PUFAs - AA (ω6 PUFA) and DHA (ω3PUFA) on haematopoiesis of sub lethally irradiated mice. We optimized the dose of the two PUFAs for maximal stimulation of haematopoiesis. Daily oral dose of 8 mg of AA/DHA was beneficial. Our results are accordance with earlier studies. Hoggatt et al. (2009) who reported that short-term ex vivo exposure of HSCs to PGE2 -a prostaglandin, derived from AA, enhances their homing, survival and proliferation, resulting in increased long-term repopulating cell (LTRC) and competitive repopulating unit (CRU) frequency. However, the
authors used PGE2 and studied in vitro effect on HSCs, whereas we report effect of in vivo feeding of purified PUFAs on haematopoiesis. Several studies suggest role of n3PUFAs or n6 PUFAs or their metabolites in stem cell proliferation (Beltz et al., 2007; He et al., 2009; Kawakita et al., 2006; Kim et al., 2009; Thangavelu et al., 2007). Our systematic study indicates a direct correlation between oral feeding of AA/DHA and stimulation of haematopoiesis in mice.

Further, we examined the effect of sub-lethal dose of irradiation on haematopoiesis in mice. A reduction in TNC count followed by drastic reduction in SP and LSK cells are hallmark effects of irradiation. Depletion in TNC count may be attributed to hampered self-renewal of HSCs, confirmed by significant reduction in SP cells and LSK percentage. Our data is consistent with earlier reports suggesting that ionizing radiation hampers HSC self-renewal and acute radiation causes BM failure (Hu et al., 2010; Lorrimore et al., 2003; Weiss et al., 2000).

We examined the effect of optimized daily dose of 8 mg of AA/DHA for ten days on sub-lethally irradiated mice and checked their effect on haematopoiesis. We observed significant increase in TNC count, SP cells and LSK cells. Enhancement in haematopoiesis may be because of protective role of PUFAs from radiation injury. Our data are in line with study done by Hoggatt et al. (2013), reporting that subcutaneous administration of PGE2 analog, to mice after irradiation, increased their survival by enhancing white blood cells (WBC), polymorphonuclear leukocytes (PMN) and platelets (PLT) over a 30 day period indicating enhanced haematopoietic recovery in mice after irradiation. Gómez de Segura et al. (2004) have reported that supplementing the diet with DHA prevented the negative action of 5-FU on mucosal morphometry in rats. Umegaki et al. (1997) noted that by feeding mice a diet containing oleic acid before X-ray exposure, experienced greater degrees of immunosuppression (53% and 69%, respectively) than did those consuming diets containing eicosapentaenoic acid alone or in combination with docosahexaenoic acid (DHA) (4% and 24%, respectively). We also observed enhancement in erythropoiesis, thrombopoiesis and leukocytes in PBL of irradiated mice fed with AA/DHA. No significant change in the number of eosinophils, neutrophils, monocytes and granulocytes was observed in PBL of fed
mice (data not shown) suggesting that oral administration of AA/DHA in mice is not causing any lineage bias. Recent study of Xia et al. (2015) showed that fish oil-rich diet promotes hematopoiesis in the bone marrow and spleen of mice by increasing TNC count, WBC count and LSK cells in part via the activity of MMP12. However in the study fish oil was mixed with the solid diet of mice. Whereas, we orally administered the defined amount of pure AA/DHA to mice.

Thus, our results demonstrate that oral feeding of AA or DHA enhances hematopoiesis in irradiated mice, and helps in partial recovery from hematopoietic injury. Further studies such as investigating radio-protectant effect of AA/DHA in the context of their ability to quench ROX species and studying mechanism of action of PUFAs will add to their therapeutic application.

Dietary interventions of AA or DHA may also enhance stem cell recovery from radiation injury, and hence indicated as an adjunct supplement to radiotherapy, for better recovery of hematopoiesis.

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CONFLICT OF INTEREST
No conflict of interest.

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