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Assessment of the bystander effect of low dose gamma radiation on rats' bone marrow

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ABSTRACT

Radiation-induced bystander effect (RIBE), is well verified in radiation biology field. Vast bulk of these effects are defined in cell culture systems, while in vivo authentication and evaluation of biological consequences still vague. We aimed to mechanistically interrogate RIBE responses thus this study was conducted. Accordingly, Albino rats' right femurs were exposed to 0.25, 0.45 and 0.75 Gy γ-radiation. Rats were euthanized 1 day and ten days post irradiation, total antioxidant capacity and mRNA expression levels of Stromal Derived Growth Factor (SDF-1) were evaluated in bone marrow. Blood micronucleated polychromatic erythrocytes (fMNPCEs) and femoral muscle's lipid peroxidation (LPO) were measurable. Femoral muscle's LPO, total antioxidant capacity and mRNA genes expression levels were significantly increased in both left and right femur at 0.45 and 0.75 Gy 24 h post irradiation and retained near control levels 10 days post irradiation. fMNPCEs became significantly higher 24 h post irradiation and showed a non-significant difference at all doses ten days post irradiation. In conclusion, the increase of LPO in femur muscle, with elevation in SDF-1 mRNA expression levels in bone marrow, and total antioxidant capacity in non-irradiated left femurs indicate that a bystander effect exists.

Introduction

Cells subjected to ionizing radiation (IR) (targeted cells) can stimulate bystander effects in non-irradiated (non-targeted cells); physically far away from the irradiated one; is a distinguished phenomenon in the area of radiation biology denoted as radiation-induced bystander effects (RIBE)[1]. *In vivo* bystander effects have been detected after radiation's clinical use [2&3]. Such effect has been investigated at high dose level in irradiated rodent models, where positron emission tomography/computed tomography (PET/CT) imaging showed that radiation-induced a rise in proliferation in the protected bone marrow contrasted to the unprotected bone marrow and controls [4].

The bystander effects changes may, technically, be either unsafe (e.g., by creating carcinogenic mutations in bystander cells) or defensive (e.g., by accelerating repair or by provoking apoptosis of damaged cells) [5]. Thus far, the huge bulk of these effects are defined in cell culture systems, while *in vivo* authentication and evaluation of biological outcomes within an organism still vague. Though humans are frequently subjected to low levels of natural IR in the environment, the health effects of low-dose ionizing radiation (LDIR) are still under investigation [6]. **Parsons *et al.*** had revealed a decline of cells in the sternal bone marrow (BM) of leukemia patients who had been subjected to X-rays distant from the place of exposure [7].

Progenitors and maturing cells from bone marrow is enhanced during urgent situations that are combined with vital needs to rapidly deal with physiological demands, such as repair and host defense. This process is called "mobilization," and it is stimulated by different stimuluses, including cytokines, chemokines, and inflammatory agents [8]. Recruitment of endothelial cells from remote locations such as the bone marrow into ischemic areas is promoted by the chemokine stromal derived growth factor (SDF-1) [9], which has been shown to be upregulated in many damaged tissues as part of the injury response [10]. SDF-1 mediates chemotactic migration of stem cells via its collaboration with chemotaxis cytokine receptor-4 (CXCR4) [11]. SDF-1/CXCR4 axis is essential for migration, apoptosis and cytokine secretion of bone marrow mesenchymal stem

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cells (BMSCs) [12]. Adult BMSCs in bone marrow have various purposes and can transfer to injured tissues and chronic inflammatory sites [13].

SDF-1/CXCR4 axis encourages the expression of SDF-1, CXCR4, nerve growth factor (NGF) through mediating BMSCs. SDF-1 draws immune cells and endogenous precursor/stem cells to the damaged site and stimulate axonal sprouting post injury [14]. Neurotropic factors such as NGF perform important functions in nerve renewal, survival maintenance, and differentiation of neurons [15]. **Chen *et al.*** implied that NGF offers a synergistic effect in the treatment of spinal cord injury in rats which encourages functional improvement and neural restoration [16]. Importantly, the SDF-1/CXCR4 axis lowered glial fibrillary acidic protein (GFAP) expression during spinal cord injury repair [17]. GFAP is expressed in the central nervous system. Lowering GFAP expression appears to mitigate astrocyte reactivation which may be useful for neuronal survival [18].

In a previous study it was suggested that exposure to X-rays may stimulate repair mechanisms, which was manifested in stimulating bone marrow production of endothelial progenitor cells (EPCs) necessary for vascular repair [19]. Accordingly, the current study sought to elucidate the role of SDF-1 in *in vivo* bystander effect of low dose gamma ionizing radiation in bone marrow of rats.

Materials and Methods

Animals

One hundred twenty male Wistar Albino rats weighing 80-120 g were obtained from the Animal Care Unit of Nile Pharmaceutical Company, El Sawah, Egypt. They were kept in plastic cages with stainless-steel grid tops in air-conditioned room with temperature maintained at 25 ± 5ºC, relative humidity and 12 h light / dark cycles. The rats were provided with a nutritionally adequate chow diet and drinking water *ad libitum.*

All care and use of laboratory animals' procedures were approved by institutional animal ethics committee for National Center for Radiation Research and Technology (NCRRT), animal's procedures were accepted in agreement with institutional standards for human care and use of laboratory animals [20].

Irradiation process

This study was conducted in NCRRT, Cairo, Egypt. The animals were exposed to partial body gamma irradiation with doses (0.25, 0.45 and 0.75 Gy) according to **Seymour and Mothersill [21]** who stated that bystander effect is owing to low dose gamma irradiation (<1 Gy). Radiation using a Gamma Cell-40 Carloirradiator, cesium 137 source with a dose rate of 26 Gy/h., in a special cage (85% attenuation) with internal dimensions of 30.5 cm in diameter by 10.5 cm deep, where the whole body was entirely shielded by a lead medical-grade shield with the right femur left unshielded and exposed to irradiation.

Experimental model

Rats were distributed, at random into seven groups:

Control group (n=24) (C): Animals were not exposed to ionizing radiation.

- **0.25 Gy group (n=24) (U1):** Animal's right femur was exposed to ionizing radiation at a dose of 0.25 Gy
- **0.25 Gy shielded group (n=8) (S1)**: Animal's whole body was protected from ionizing radiation of dose 0.25 Gy to ensure the attenuation of lead shield against 0.25 Gy radiation.
- **0.45 Gy group (n=24) (U2):** Animal's right femur was exposed to ionizing radiation at a dose of 0.45 Gy
- **0.45 Gy shielded group (n=8) (S2):** Animal's whole body was protected from ionizing radiation of dose 0.45 Gy to ensure the attenuation of lead shield against 0.45 Gy radiation.
- **0.75 Gy group (n=24) (U3):** Animal's right femur was exposed to ionizing radiation at a dose of 0.75 Gy radiation.
- **0.75 Gy shielded group (n=8) (S3):** Animal's whole body was protected from ionizing radiation of dose 0.75 Gy. to ensure the attenuation of lead shield against 0.75 Gy radiation.

For each group half of the rats were dissected under anesthesia after one day and the other half after ten days. Bone marrow and muscle femur were collected from both irradiated (right) and non-irradiated (left) femurs and subjected to further analysis.

Sample preparation:

Under aseptic, femur was dissected, and bone marrow was flushed out with phosphate buffered saline solution (pH=7.4). Bone marrow cells were gathered by centrifugation (i.e. 1000-2000 rpm for 10min. at 4ºC). Cell pellets were homogenized in 1-2 ml of ice-cold buffer (i.e. 5mM Potassium phosphate, PH=7.4, containing 0.9 % sodium chloride and 0.1 % glucose). Then it was centrifuged at 4000 rpm for 15 min. at 4ºC. The supernatant was removed and stored on ice till used. Blood samples were acquired by cardiac puncture and drawn into a heparinized centrifuge tube. Femoral muscle tissue homogenate was prepared by grinding tissue in liquid nitrogen followed by homogenization in PBS.

Biochemical Investigations

Measurement of oxidative stress status

Bone marrow and muscle femur collected from all animal groups were subjected to lipid peroxidation (LPO) determination according to **Yoshioka *et al*** [22]. Malondialdehyde (MDA) concentration was applied as the indicator of LPO. It was established by determining thiobarbituric acid (TBA) reactive species. The absorbance of the resultant pink product was measured at 532 nm.

Also, total antioxidant capacity was evaluated in bone marrow by colorimetric technique using Biodiagnostic commercial kit (diagnostic and research reagents cat no.

TA 25 13, Egypt). The determination of the antioxidant capacity is achieved by the reaction of sample's antioxidants with a specified amount of exogenously supplied hydrogen peroxide (H_2O_2). The antioxidants in the sample reduce a particular quantity of the supplied hydrogen peroxide. The remaining H_2O_2 is measured colorimetrically by an enzymatic reaction which necessitates the conversion of 3,5 dichloro-2-hydroxybenzensulphonate to colored product. The absorbance of the resultant color was measured at 532 nm.

Quantitative Real Time Polymerase Chain Reaction (QPCR)

According to instructions of the Thermo Scientific Gene Jet RNA purification kit, total RNA was obtained from bone marrow samples. The concentration and purity of the total RNA samples were acquired using a Nanodrop ND-1000 spectrophotometer. Total RNA was then used for cDNA synthesis using Thermo Scientific Revert Aid First Strand cDNA Synthesis Kit according to the manufacturer's recommendations.

Real-time PCR (qPCR) was carried out using the reaction mixture of 1 µl cDNA, 0.5 mM of primer Stromal Derived Growth Factor (SDF-1) and Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) as internal control), Thermo Scientific Maxima SYBR Green/ROX qPCR Master Mix (2X) in a total volume of 25 µl, according to manufacturer's protocol. The qPCR tubes were run on Stratagene (Mx3000PTM) machine and the results were computerized. The primer sequences are shown in **Table 1.**

Data Analysis was done using the ΔΔCT method of relative quantification **[23]**. The SDF-1 expression levels were normalized to GAPDH and shown as fold change relative to controls and were calculated as $2^{(-\Delta\Delta CT)}$. Where ΔCT = CT target gene – CT reference gene and ΔΔCT = ΔCT (experimental sample) − ΔCT (control sample).

Micronucleus assay

Micronuclei assay was carried out in blood according to **Darzynkiewicz [24].** Whole blood smears were set on clean microscope slides. Blood smear slides were air dried, methanol fixed and stained with acridine orange (2 mg/ml distal water). Before scoring, slides were let to stand for a few hours to allow cells to settle and to maximize staining then examined by fluorescence microscopy at ×400. Micronucleated immature erythrocytes stained bright red/orange were scored. The frequency of MNPCEs was determined using 100 PCE per field of each slide per animal.

Statistical analysis

Statistical analysis was conducted by digital computer assistance, using Excel and the Statistical Package for Social Science (SPSS software package, version 20.0) programs (SPSS Inc., Chicago, IL, USA).

Data are shown as means ± standard error (S.E). Statistical differences between groups were estimated by one-way analysis of variance (ANOVA) followed by multiple comparison testing used to evaluate variations between individual of means among groups. *P*-value < 0.05 is significant.

Table 1: Primer sequence of QPCR genes

Results

Oxidative stress status among different groups

Radiation produced a significant rise in lipid peroxidation 24 h post irradiation in both left and right muscle femur compared to control group and shielded groups at doses 0.45 and 0.75 Gy, while non-significant difference was observed at dose 0.25 Gy **Fig. 1A**. Ten days post irradiation, level of LPO in both left and right femurs retained near control level together with shielded group at all doses, ($p > 0.05$) **Fig. 1B**.

Radiation made a significant rise in total antioxidant capacity in bone marrow in both left and right femurs at doses 0.45 and 0.75 Gy compared to control group and shielded groups, while non-significant difference was detected at 0.25 Gy 24 h post irradiation **Fig. 2A**. After 10 days, there was non-significant difference in total antioxidant capacity in both left and right femurs compared to control group at all doses together with shielded groups **Fig. 2B**.

For all measured parameters non-significant changes were noted between control group and shielded groups at all doses. Among all groups, there was a non-significant difference between left and right femur at all doses.

Levels of mRNA gene expression among different groups

Radiation induced significant increase in SDF-1 mRNA expression levels compared to control and shielded groups at doses 0.45 and 0.75 Gy, while non-significant difference in all genes except GFAP was observed at dose 0.25 Gy 24 h post irradiation **Fig. 3A**.

After 10 days, a significant increase in mRNA expression levels of SDF-1 were shown in both left and right femurs compared to control groups at doses 0.45 and 0.75 Gy, while dose 0.25 Gy showed non-significant difference when compared with control groups **Fig. 3B** .

For all measured parameters there were non-significant variations between control group and shielded groups at all doses.

Frequency of micronucleated polychromatic erythrocytes (fMNPCEs) among different groups

Immature erythrocytes cytoplasm contains RNA and could be differentiated simply from mature erythrocytes, which do not fluoresce since they lack RNA. Micronuclei is the only organelle that holds DNA in the mammalian erythrocyte, and it can consequently be detected obviously and specifically. Twenty-four hours and ten days post irradiation significant increase of blood fMNPCEs in all doses in non-shielded exposed rats compared to control group and shielded groups and it was highest at 0.75 Gy, see **Fig. 4A&B**. For all measured parameters there were non-significant differences between control group and shielded groups at all doses.

Gene	Forward Primer sequence	Reverse Primer sequence
SDF-1	5'GCCCCTGCCGATTCTTTGAG3'	5'GTCCAGGTACTCTTGGATCCAC3'
GAPDH	5'CAAGGTCATCCATGACAACTTTG3'	5'GTCCACCACCCTGTTGCTGTAG3'

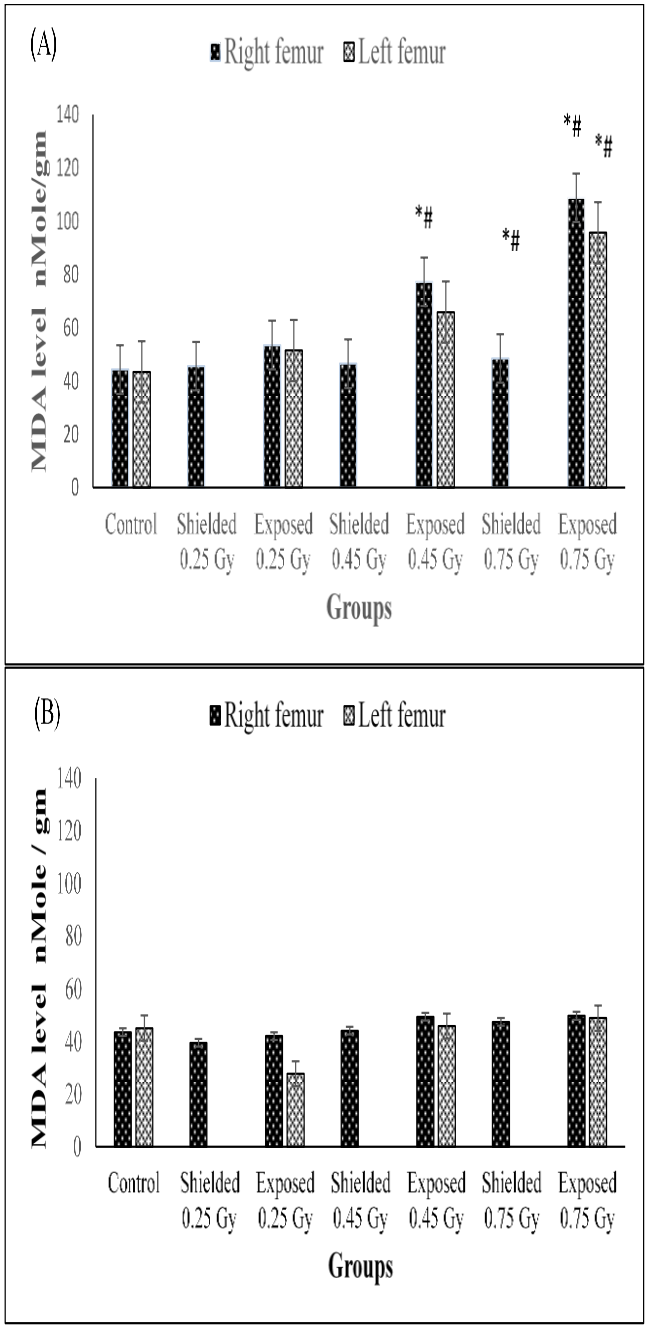

Fig. 1: Levels of MDA (nMole/gm) in femur muscle (**A**) 24 hours post irradiation (**B**) 10 days post irradiation among different groups. *****$p < 0.05$ is significant compared to shielded group, $^{\#}$ $p < 0.05$ is significant compared to control group.

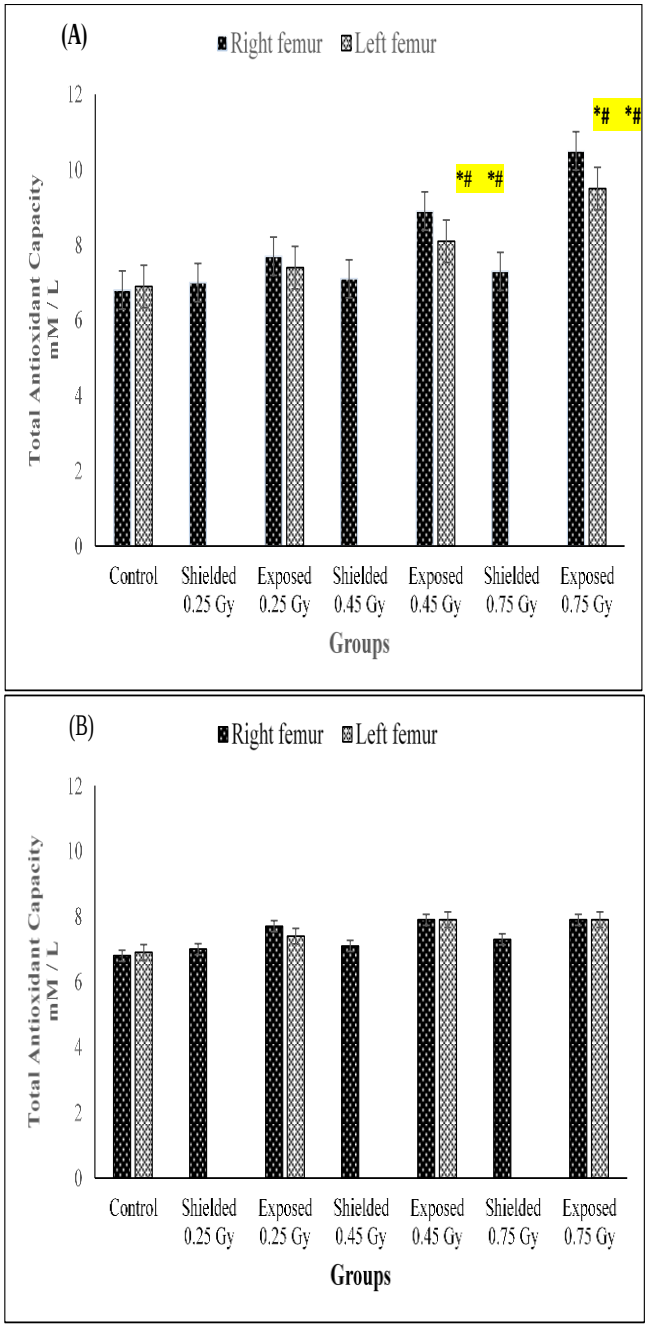

Fig. 2: Levels of total antioxidant capacity in bone marrow (**A**) 24 hours. post irradiation (**B**) 10 days post irradiation among different groups. *****$p < 0.05$ is significant compared to shielded group, $^{\#}$ $p < 0.05$ is significant compared to control group.

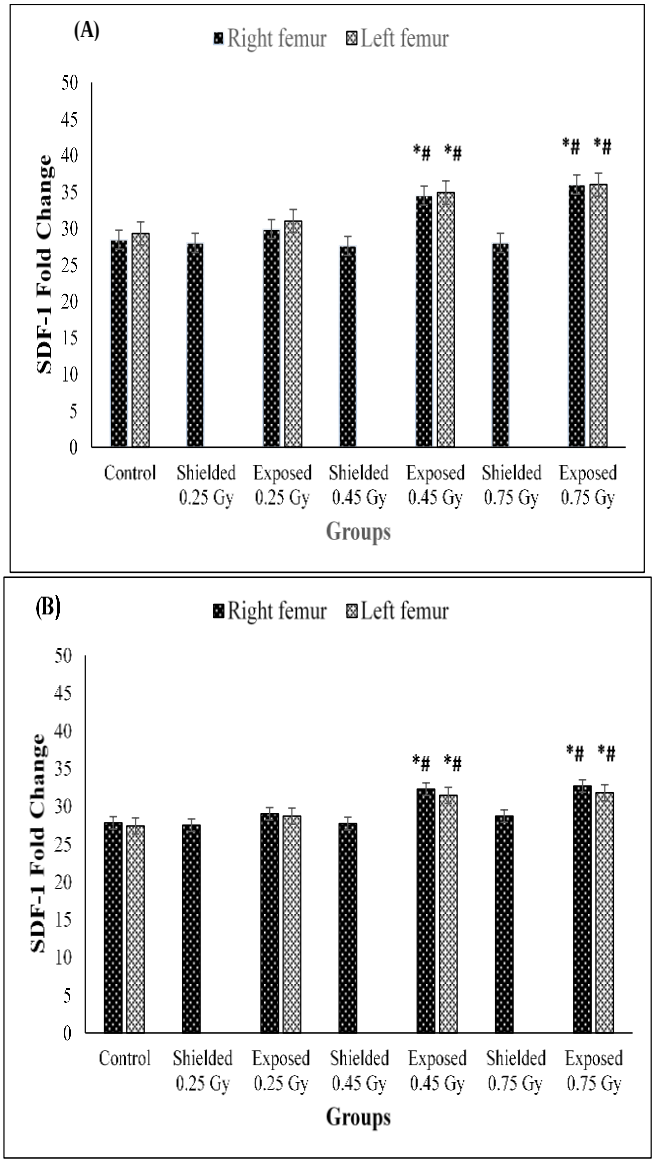

Fig. 3: Level of mRNA expression of SDF-1 in bone marrow (A) 24 hours post irradiation (B) 10 days post irradiation among different groups. *****$p < 0.05$ is significant compared to shielded group, $^{\#}$ $p < 0.05$ is significant compared to control group.

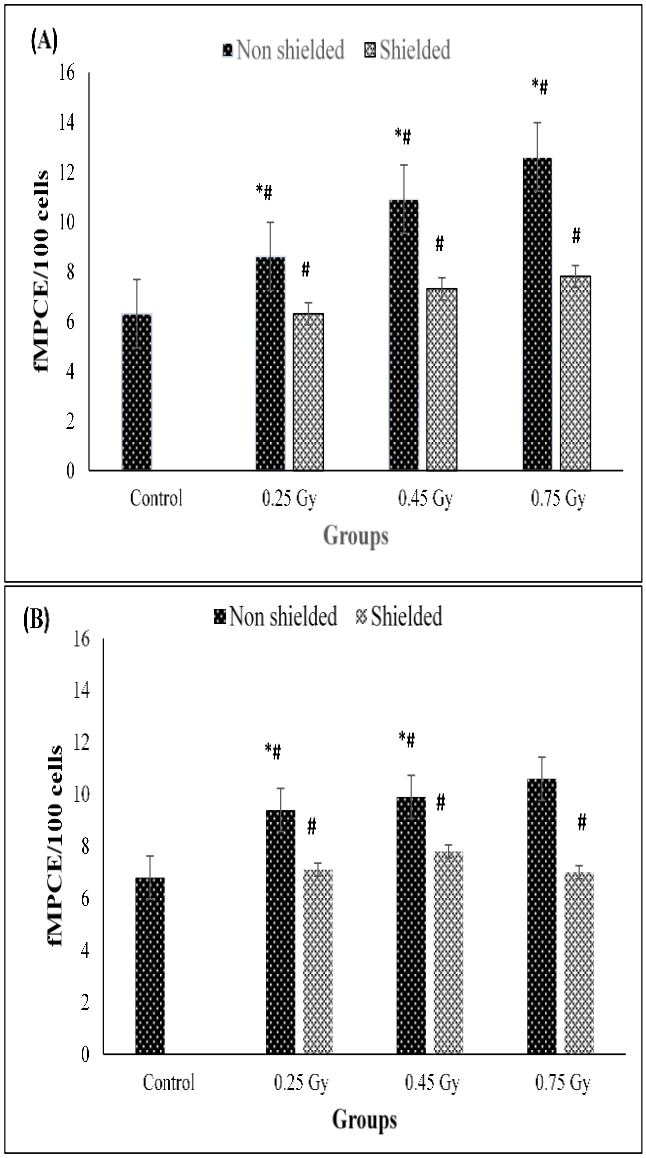

Fig. 4: fMPCEs in blood **(A)** 24 hours post irradiation **(B)** ten days post irradiation among different groups. *****$p < 0.05$ is significant compared to shielded group, $^{\#}$ $p < 0.05$ is significant compared to control group.

Discussion

Bystander effect is biological changes in non-irradiated cells by diffused signals from irradiated bystander cells, which produces the radiation toxic effects on the adjacent non-irradiated tissues. It has been assumed that bystander cells, by producing bystander factors, are actively implicated in the propagation of bystander effect in the regions away from the initial irradiated site [25]. Accordingly, in the present study we investigated bone marrow mRNA expression levels for SDF-1 gene as a measure for IR-induced repair with antioxidants levels for adaptive response concomitant with blood fMNPCEs as a marker for IR damage.

Membrane lipids are the primary target to IR-induced free radicals [26]. Lipid peroxidation is a master molecular mechanism implicated in the cell structures oxidative damage and in the cell death toxicity approaches [27]. Primarily, the present study detected no effect of IR in LPO in bone marrow cells at the applied doses of 0.25, 0.45 and 0.75 Gy γ-radiation. This finding provides further proof to a previous study which concluded that in case of entire-body irradiation radicals' scavengers and other metabolic reactions could inhibit a quantifiable rise in LPO [28].

Ionizing radiation facilitated the generation and accumulation of mitochondrial reactive oxygen species (ROS) [12]. Accordingly, the current study measured lipid peroxidation in femur muscles. The ongoing study shows a significant rise in LPO in both left and right femurs compared to control and shielded group at doses 0.45 and 0.75 Gy after 24 h and this increase retained near control after 10 days of irradiation. **Zhang *et al*** [29] showed that IR stimulates the release of ROS and the cellular response to irradiation depends on a wide variety of factors. Increase in the level of ROS is the most important factor. **Kojima *et al.* (1998a, 1998b)** [30&31] stated that post irradiation at low levels the lipid peroxidation levels in the brain, thymus and bone marrow showed persistent decreased within various period of time, which agree with the current results.

Antioxidants are the first line of defense against oxidative damage and functions as free radical scavenger [32]. A study by **Waer and Shalaby** [33] showed that an antioxidant defense mechanism is employed to keep redox balance, and that proper antioxidants may decrease toxicity of free radicals and keep from radiation damage. In this study, radiation produced a significant increase in total antioxidant capacity in both left and right femurs at doses 0.45 and 0.75 Gy compared to control group and shielded groups, while non-significant difference was observed at 0.25 Gy, 24 h post irradiation. [34] **Scott *et al.* (2009**) supported the viewpoint that introduction to low radiation doses (mild stresses) increase protective antioxidants which may contribute to hermetic phenotypes.

After 10 days, there was non-significant difference in total antioxidant capacity in both left and right femurs compared to control group at all doses together with shielded groups, but significant decrease at 0.45 and 0.75 Gy when compared to their levels 24 h after irradiation. Results are in agreement with earlier records that antioxidant markers such as superoxide dismutase (SOD) activities in bone marrow was persistent after radiation-induced rise for about 1 week [35].

Stromal cell-derived factor-1 (SDF-1) is an endothelial progenitor cell (EPC) chemokine established to be in control for both progenitor cell mobilizations from the bone marrow to peripheral blood and resting to the places of tissue and vascular injury [36]. Data regarding effect of LDIR on bone marrow expression of SDF-1 are scarce. However, animal experiments have demonstrated that IR rises stem cell–active mobilization factors as it stimulates a novel pathway stimulating EPCs migration directly through the expression of SDF-1[37].

Inflammation owing to irradiation can mobilize hematopoietic stem cells into the circulation; irradiation can promote molecular pathways that enhance the discharge of tissue chemokines, which draw stem cells to tissues where they may rest and differentiate [2].

Accordingly, increase in mRNA expression levels of SDF-1 compared to control and shielded groups at doses 0.45 and 0.75 Gy, can be regarded as repair response to radiation induced damage and it can be speculated that SDF-1 gene will mobilize specific progenitor stem cells to the site of damage to initiate repair.

In this study radiation stimulated a significant increase in SDF-1 in both left and right femurs compared to controls at doses 0.45 and 0.75 Gy 24 h post irradiation. At 0.25 Gy there was a non-significant difference in SDF-1 compared to controls. In agreement with us *in vitro* findings by **Peled *et al.*** [38] showed that following a sub lethal dose of ionizing radiation, the SDF-1 mRNA expression levels increased significantly 24 and 48 hours after irradiation. Ten days post irradiation there was a significant decrease in SDF-1 mRNA expression level in both left and right femurs at doses 0.45 and 0.75 Gy compared with their levels at 24 hours post irradiation. This indicates that after regenerating damage, SDF-1 mRNA returns to control level.

Micronucleus test is a cytogenetic method for evaluating cytotoxic effects as ionizing radiation and chemical materials in mammalian system [39]. Thus, we used it to assess cytogenetic effects of gamma radiation on the proliferation of the bone marrow cells. Results revealed that frequency of micronucleated polychromatic erythrocytes (fMNPCEs) significantly increase 24 h post irradiation at all doses and was highest at 0.75 Gy in non-shielded exposed rats compared to control and shielded groups. These findings agreed with former reports, where micronucleus formation due to irradiation has been described in the bone marrow polychromatic and normochromatic erythrocytes which were examined at 24 h post irradiation at low doses. Ten days post irradiation fMNPCEs showed non-significant difference for all doses when compared to their level at 24 h post irradiation. The observed decrease after 10 days compared to their levels post 24 h can be attributed to the fact that spleens can remove micronuclei from the peripheral circulation [40].

In spite of massive research showing the phenomenon of RIBE in numerous biological systems and detection of many agents implicated in inter-cellular signaling, the mechanism(s) responsible for RIBE are yet not fully realized [41]. Inter-cellular gap-junctional communication or soluble factors discharged from irradiated cells have been involved in RIBE [42]. Reactive oxygen species (ROS) [29] and cytokines, such as transforming growth factor beta (TGF-β) [43] and tumor necrosis factor α (TNF-α) [44] have also been considered to be involved in RIBE.

Other reports suggested that miRNAs perform an essential role in inter-cellular signaling between irradiated and bystander cells [45]. For example, prevention of miR-27b may upregulate SDF-1α protein expression and eventually supports mesenchymal stem cell directional movement and damage recovery [46].

In conclusion the increase in LPO level of femur muscle, mRNA expression level of SDF-1 in bone marrow and total antioxidant capacity in both left and right femurs showed that a bystander effect exists *in vivo*. This indicates the possible role of bystander effect in causing expression of genes in tissues that were not exactly subjected to ionizing radiation. It is clear that after irradiation, cells react with rise in the expression of cellular antioxidant defenses, demonstrating one of the extremely powerful mechanisms of preventing impairment. Such an improvement of the antioxidative capacities plays an essential part in the decline of initial lipid peroxidation and DNA damage by low-dose-rate radiation. IR stimulates DNA damage 24 h post irradiation and also stimulates tissue regeneration 10 days post irradiation which is manifested by fMNPCEs. Additional researches are necessary to explore the role of regulatory network of cytokines and miRNAs in controlling SDF-1 genes involved in bystander effect.

Conflict of Interest

Authors declare no interest conflict. This research did not receive any fund.

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