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Assessing Radiosensitivity: Effects of Acute Ionizing Radiation on Inflammation and Apoptosis in Macrophage Cell Line (RAW 264.7)

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Abstract

Introduction: The responses of biological systems to various types of radiation have multifaceted dimensions. In the field of ionizing radiation, in vitro external gamma radiation therapy has primarily been studied as a model to elucidate the challenges that biological systems face from radiation effects. Exposure of cells/organisms to gamma radiation results in a cascade of ionization events that can cause severe and irreversible biological damage. However, the biological responses and oxidative stress-related mechanisms under acute radiation conditions remain poorly understood in inflammatory systems. The present study aimed to provide a model of the effect of ionizing radiation on macrophages, which play a pivotal role in the mechanisms of inflammation, to assess the impact of radiotherapy as an approach to treating inflammatory diseases.

Methods: A macrophage cell line (RAW 264.7) was cultured and exposed to different doses of gamma radiation (4, 6, 8, 10 Gy). Cell viability, apoptosis, cell cycle, migration, nitric oxide (NO) and prostaglandin E2 (PGE2) production, expression of pro-inflammatory and apoptotic genes, and cytokine secretion of macrophages were also evaluated.

Results: The results showed that gamma radiation at 4 Gy had a low effect on macrophage characteristics and cytokine secretion patterns. In contrast, higher doses (8 and 10 Gy) increased DNA damage, expression of apoptotic genes, and secretion of NO and PGE2 cytokines. 6 Gy radiation, the maximum radiation dose, showed moderate non-destructive effects and inflammation process modulation. In this study, doses higher than 6 Gy of Gamma radiation caused cell mortality.

Conclusion: It appears that 6 Gy of gamma radiation modulates the inflammatory cascade caused by macrophage cells.

Keywords: Macrophage; Gamma radiation; Inflammation; Apoptosis.

Introduction

The immune system responds to destructive stimuli, such as damaged cells, pathogens, toxins, and irradiations, by initiating inflammation, which removes harmful agents and initiates the healing process. However, uncontrolled acute inflammation can become chronic and cause a range of chronic inflammatory disorders and pathophysiology.[1-3] Inflammation is a common effector mechanism that leads to extracellular matrix remodeling, fibrosis, angiogenesis, oxidative stress, and tissue damage in different organs.[4] Acute and chronic inflammation responses in the pancreas, kidney, heart, brain, intestinal tract, reproductive system, liver, and lung cause inflammatory diseases such as atherosclerosis, arthritis, inflammatory bowel disease, and kidney and lung disease.[4,5]

Inflammatory cytokines such as tumor necrosis factor-alpha (TNF-alpha) and interleukin-6 (IL-6), and IL-1β are biomarkers used for inflammatory disease prognosis, diagnosis, and therapeutic decisions.[6-10] Monocytes, macrophages, and other cells are responsible for local responses to infection and tissue damage. Monocytes can differentiate into macrophages and dendritic cells and are attracted to damage sites via chemotaxis. Most disorders,

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including asthma, cancer, diabetes, atherosclerosis, chronic inflammatory disease, and degenerative and autoimmune diseases, are related to the alterations of inflammation-mediated immune cells.[11-13]

According to the extensive use of ionizing irradiation in medicine, low doses or acute ionizing irradiation is a new approach that is widely under study and investigation. However, although its therapeutic effects are not clearly evident, its harmful biological effects cannot be neglected.[14] It has been shown that radiation can cause an inflammatory response and has effects on the expression of the ZEBRA protein and stress mediators (glucocorticoid) in the Epstein-Barr virus (EBV). EBV reactivation induced by 2 and 4 Gy gamma irradiation caused a combination of glucocorticoid and gamma radiation that plays a key role in the inflammatory response.[15]

In 2010, researchers reported that exposure to ionizing radiation showed a dose-dependent increase in the expression of IL-6 at 20 hours, while immediately after radiation, the release of IL-8 decreased compared to the control group but elevated over time, particularly at a 0.5 Gy radiation dose.[16] As previously reported, 0.5 Gy gamma irradiation upregulated MKP-1 and suppressed TNF-α production by P38 MAPK inactivation in mouse macrophage cell lines.[17] In addition, other researchers have shown that a low dose of gamma radiation increases the expression and activation of Nrf2 in the macrophage RAW264.7 cell line.[18] In 2018, the enhancement of INF/IL4 in lymphocytes of BALB/c spleen by a low dose of local gamma radiation suggested that acute ionizing radiation might be a good choice in radiotherapy and could attenuate inflammatory disease. The purpose of this study was to evaluate the effect of different doses of gamma radiation (4, 6, 8, 10 Gy) on cell viability, apoptosis, cell cycle, migration, and motility of RAW264.7 macrophage cells. Furthermore, cytokine production, nitric oxide (NO) and prostaglandin E2 (PGE2) production as inflammation mediators, and expression and upregulation of pro-inflammatory and apoptotic genes were investigated to provide a comprehensive model of acute gamma radiation on the macrophage cell line, which is the main mediator of all inflammatory responses.

Materials and Methods

Cell Culture

Macrophage cell line RAW 264.7 was obtained from the National Cell Bank of Iran (NCBI). The cells were cultured in Dulbecco's Modified Eagle medium (DMEM) (GIBCO) supplemented with 10% fetal bovine serum (FBS) (GIBCO, Life Technologies, Ghent, Belgium), 100 U/mL of penicillin, and 100 µg/mL of streptomycin. The cells were grown until they reached 70–80% confluence in the culture flask (Figure 1a). The cells were passaged and plated at 1:3 or 1:2 dilutions every 2–3 days using 0.25% trypsin and 1mM EDTA (Invitrogen LT, Merelbeke, Belgium). The cells were frozen in 90% DMEM with 10% DMSO (Merck, Darmstadt, Germany) in liquid nitrogen.

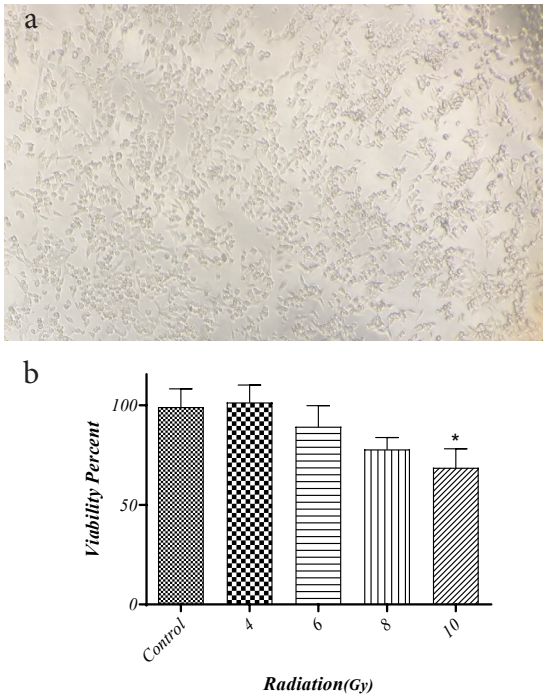

Figure 1. RAW264.7 Cell Survival After 24 Hours Following Exposure to 4, 6, 8, and 10 Gy of Gamma Radiation Relative to the Untreated Control, Measured by MTT Assay. **(a)** RAW264.7 cells were cultured in DMEM medium (×10). **(b)** MTT assay for the Raw264.7 line with a different gamma dose: change in viability percent with an increasing irradiation dose. The obtained results are expressed as mean ± SD of at least three replicates (* $P \leq 0.05$)

Cell Viability Screening by MTT Assay

RAW 264.7 macrophage cells were seeded in 96-well plates (1×10^4 cells/well) and irradiated in the Radiotherapy Department of Imam Hospital at room temperature, using a Co-60 gamma-ray source (Source Canada, Theratron 780 E) with a dose-rate of 0.4 Gy/min., at a distance of 1 m from the source. This study was aimed at investigating the effect of gamma radiation with 4, 6, 8 and 10 Gy doses. In the control group, the cells received no radiation.

Cell viability was measured by MTT (3-(4, 5-Dimethyl-2-thiazolyl)-2, 5- diphenyl-2H-tetrazolium bromide) assays, 24 hours after radiation, as described previously.[19] The percentage of cell viability was calculated and compared to the control (100% of viability).

Apoptosis Process Assay by Flow Cytometry

RAW 264.7 macrophage cells seeded in 6-well plates (16×10^4 cells/well) and irradiated with at least 4, 6, 8 and 10 Gy doses. Other irradiation conditions were done in the same way as the previous assay. The control group received no radiation. 24 hours after the treatment, the cells were collected in 500 µl Binding Buffer (Annexin V-FITC/PI apoptosis detection kit, Jiangsu Keygen Biotechnology). Annexin V fluorescein isothiocyanate

and propidium iodide (FITC; 5 µL) were added to the cell suspension and incubated for 15 min at room temperature in the dark. The cells were analyzed with the FACSCalibur flow cytometer via the BD CellQuest™ Pro Analysis system (BD Biosciences, USA).

Cell Cycle Analysis by Flow Cytometry

After collection, the cells were seeded in 6-well plates and the cell pellet was washed twice with a PBS buffer and fixed in a cold 70% EtOH solution at 4 °C for 24 hours. The fixed samples were washed with PBS and stained with a 1 ml propidium iodide cocktail (50 µg/mL PI + 20 µL RNase A + 1 µL triton x-100) for 30 min. at room temperature. Afterwards, the samples were subjected to cell cycle description by flow cytometry (BD Biosciences, USA). The cells in G0/G1, S, and G2/M-phase were determined by filtering for doublets and aggregates.

In Vitro Migration Assay

For this assay, 4×10^4 cell/well was seeded onto 24-well plates. After overnight incubation at 37 °C and 5% CO2, the cells were starved for 24 hours. The cells were cultured and grown to 90% confluency and subjected to 1% mitomycin C at 37 °C for 8 hours to stop cell proliferation. The cell monolayers were scratched horizontally with a P100 pipette tip. The cells were gently washed twice with PBS to remove cell debris. The monolayer was rinsed three times with PBS and placed in DMEM with 1% FBS followed by irradiation (4, 6, 8 and 10 Gy). Reduction in the scratch areas was monitored by an inverse light microscope (ECLIPS 80i with WG filter, Nikon, Tokyo, Japan) equipped with a digital camera. The images were taken at the interval time of 0, 24, and 48 hours.[20] The percentage of migration was calculated based on the reduction in the scratch area measured at the specific time as described below:

$$= \frac{scratch\ area\ 0 - scratch\ area\ at\ specific\ time}{scratch\ area\ 0\ h} \times 100$$

Invasion Assay

The 24-well trans-well invasion assay was performed to measure the invasion of the cells under treatment conditions. The cells (4×10^4 cell/well) in DMEM supplemented with 1% FBS cultured in the upper chamber with 8 µm pore (SPL life science Co., Gyeonggi-do, Republic of Korea). The cells were allowed to adhere for 2 hours and then exposed to irradiation (4, 6, 8 and 10 Gy). After 24-hour rest in the incubator, the polycarbonate membrane was removed. The cells that migrated through pores to a lower surface were rinsed with PBS, fixed in 100% methanol, and stained with ethanol-based crystal violet solution. Relative to the control, the migrated cells were shown as the means of the cell numbers in fifteen randomly selected visions.

Stimulation Inflammatory Pathways of Raw264.7 Cells With LPS

Lipopolysaccharide (LPS) (1 µg/mL) was used to stimulate and activate inflammatory pathways in Raw264.7 Macrophage cells. The cells were incubated for 18 hours with LPS at 37 °C, 5% CO2 and 95% humidity.

Cytokine Releases the Assay by ELISA

Determined by ELISA kits (BD Biosciences, San Jose, CA, USA), cytokines were secreted from the cells to the media, according to the manufacturer's instructions. Briefly, RAW264.7 macrophage cells (2×10^4 cells/well) were cultured in a 48-well plate with DMEM containing 10% FBS, incubated at 37 °C and 5% CO_2 incubator. The content level of IL-1β, IL-6 and TNF-α in culture media was evaluated at 450 nm in a BioTek ELx808 microplate reader (control, LPS and irradiated with 4, 6, 8 and 10 Gy groups). The absorbance values were converted to concentrations of IL-1β, IL-6, and TNF-α (pg/mL) using standard curves prepared with serial dilutions of recombinant IL-1β, IL-6, and TNF-α standard protein.

NO And PGE2 Production

The cells (16×10^4 cell/mL) were cultured in 6 well plates and irradiated (4, 6, 8 and 10 Gy). After 24 hours, cell rest from the irradiation, measured the quantity of nitrite as an indicator of NO release to the culture medium. The level of nitrite – stable metabolite of NO – was measured using the Griess reagent (1% sulfanilamide and 0.1% naphthylethylenediamine dihydrochloride in 2.5% phosphoric acid). Briefly, 100 µL of the cell culture medium was mixed with 100 µL of the Griess reagent. Subsequently, the mixture was incubated at room temperature for 10 minutes, and then the absorbance was measured at 540 nm by a microplate reader. A fresh culture medium was used as a blank in every experiment. The quantity of nitrite was determined using the sodium nitrite standard curve. The concentration of PGE_2 in cell culture media was determined using a PGE_2 assay ELISA kit (Cayman Chemicals, Ann Arbor, MI, USA) following the manufacturer's instructions.

Quantitative Analysis of Gene Expression

Total RNA was extracted from each one of the groups (control, LPS and irradiated with 4, 6, 8 and 10 Gy groups) using the RNeasy Mini Kit (Qiagen, USA) according to the manufacturer's recommendations. The quantity and quality of extracted RNA were determined by UV spectrophotometry (Eppendorf, Germany). cDNA synthesis was performed from 500 ng DNase-treated RNA samples with a QuantiTect Reverse Transcription Kit, using oligo (dT) primers. The specific primers were used for PCR reactions (Table 1). Quantitative polymerase chain reaction (q-PCR) reaction was performed using master mix and cyber green in an Applied Biosystems,

Table 1. Sequence of Primers Used in This Study

Name	Forward	Reverse	Length
P53	5-ACC GCC GAC CTA TCC TTA CC-3	5-TCT TCT GTA CGG CGG TCT CTC-3	118
BCL2	5-GTCCCGCCTCTTCACCTTTCAG-3	5-GATTCTGGTGTTTCCCCGTTGG-3	148
BAX	5-TGA AGA CAG GGG CCT TTT TG-3	5-AAT TCG CCG GAG ACA CTC G-3	140
GAPDH	5-TGG ATT TGG ACG CAT TGG TC-3	5-TTG CAC TGG TAC GTG TTG AT-3	210
TNF α	5-TCA AAC CCT GGT ATG AGC CC-3	5-ACC CAT TCC CTT CAC AGA GC-3	154
IL-1β	5-GAA ATG CCA CCT TTT GAC AGT G-3	5-TGG ATG CTC TCA TCA GGA CAG-3	116
IL-6	5-ACG GCC TTC CCT ACT TCA CA-3	5-CAT TTC CAC GAT TTC CCA GA-3	129
iNOS	5-AAC GGA GAA CGT TGG ATT TG-3	5-CAG CAC AAG GGG TTT TCT TC-3	147

StepOne™ thermal cycler (Applied Biosystems, USA). The PCR program started with an initial melting cycle for 5 minutes at 95 °C to activate the polymerase, followed by 40 cycles of melting (30 seconds at 95 °C), annealing (30 seconds at 58 °C) and extension (30 seconds at 72 °C). The quality of the PCR reactions was confirmed by melting curve analyses. The efficiency of primers was determined for each gene using a standard curve (logarithmic dilution series of cDNA of each sample). The reference gene (GAPDH) and target genes were amplified in the same run. The target genes were normalized by the reference gene (that had equal expression in all runs) and expressed relatively to a calibrator.

Statistical Analysis

Data were analyzed by one-way analysis of variance (ANOVA) followed by Tukey post hoc test using GraphPad Prism 8.0 (San Diego, USA). All obtained data were expressed as mean ± SD of three replications. Data with *P* values lower than 0.05 were considered statistically significant.

Results

Radiation Causes Changes in Viability

The effect of radiation on the viability of a macrophage cell line was investigated using the MTT assay. After a 24-hour rest period following exposure to 4, 6, 8, and 10 Gy of gamma radiation, the cytotoxicity of each dose of radiation was assessed. The results showed that the viability of RAW 264.7-treated cells was not affected by radiation doses less than 10 Gy. At this dose of radiation, the viability significantly increased ($P \leq 0.05$) (Figure 1b).

Radiation Increases Apoptosis in the Raw264.7 Cell Line

The rate of apoptosis in the radiation-treated macrophage cells (the lower right quadrant) significantly increased in all treated groups (Figure 2) by Flow cytometry analysis. Therefore, the percentage of live cells significantly decreased in all treated groups except the 4 Gy radiation group. The high-dose exposed groups (8 and 10 Gy) showed an increase in apoptosis percentage (Figure 3a) ($P \leq 0.001$).

Gamma Radiation Induces G2/M Arrest in the Macrophage Cell Line

The influence of gamma radiation on cell cycle progression and distribution was analyzed using flow cytometry. The results indicated that the arrest of cells in the G2/M phase increased in all treatment groups (Figure 4). This inhibition was greater in the 8 and 10 Gy groups (Figure 3b).

In Vitro Migration Assay

The evaluation of the effect of gamma radiation on the migration potential of macrophages was assayed using the scratch method, which mimics to some extent the migration of cells in vivo. The monolayer culture of Raw264.7 treated with different doses of gamma radiation showed a significantly lower migration potential rate compared to the control group after 0, 24, and 48 hours (Figure 5b). This finding indicates that gamma radiation reduces the migration potential of macrophage cells (Figure 6).

Gamma Radiation Increases Cell Invasion in Raw264.7 Cells

Another important characteristic of the biological effects of gamma radiation involves its effect on cell invasion. In order to minimize cell division, the cells were subjected to a starvation period by the supplementation of the culture media with only 1% FBS. The trans-well invasion assay illustrated three-dimensional movement, where macrophage cells were cultured in the upper chamber of 8 μm pore size trans-wells, allowed to adhere for 2 hours, and then irradiated. The cells were then incubated for 24 hours, and the migrated cells were counted at the end of the rest time. The results showed that gamma radiation significantly increased the ability to migrate and the mobility of the cells (Figure 5a).

Inhibitory Effect of Gamma Radiation on Pro-inflammatory Cytokines

To determine whether gamma radiation might also have effects on the modulation of pro-inflammatory cytokines in RAW264.7 macrophage cells, the pro-inflammatory cytokines, such as IL-1β, IL-6, and TNF-α, were

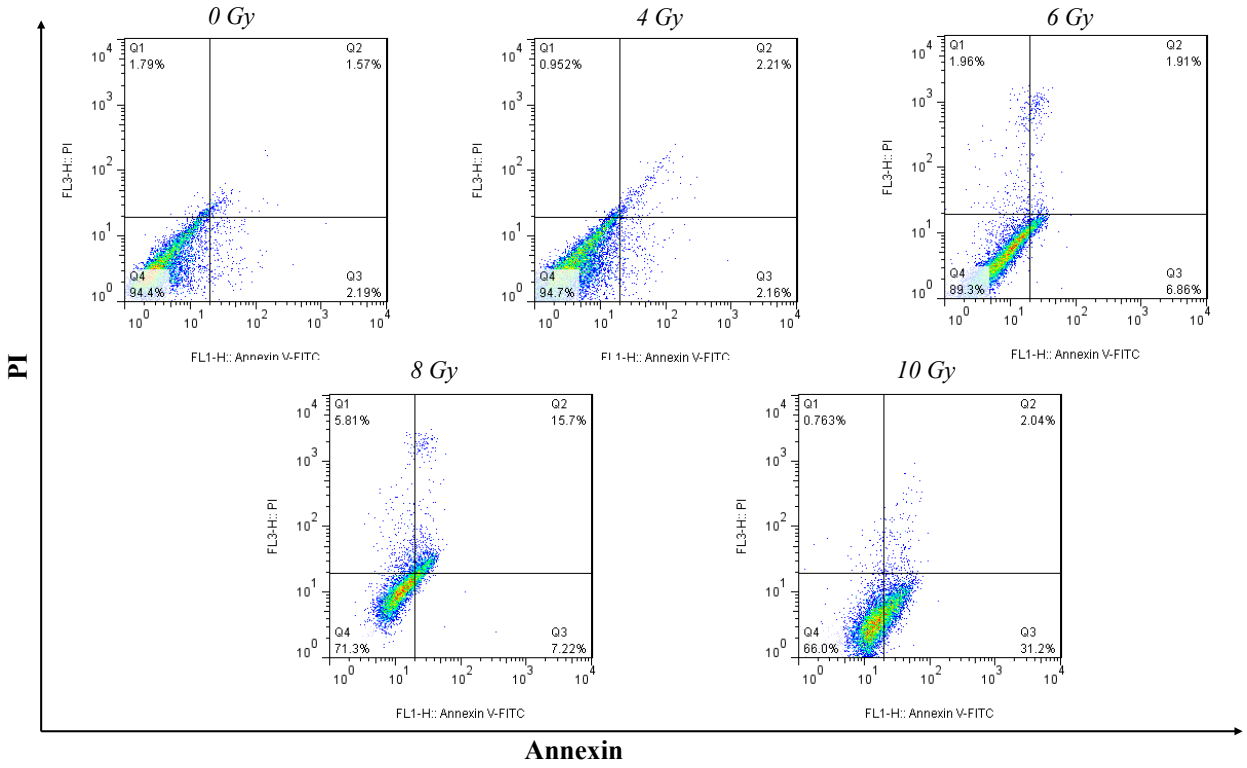

Figure 2. Dot Plot of Apoptosis Assays by Annexin V and PI Method in Cells Treated With Gamma Radiation After 24 Hours. Q4; live cells, Q3; early apoptosis cells, Q2; late apoptosis cells, Q1; necrosis cells

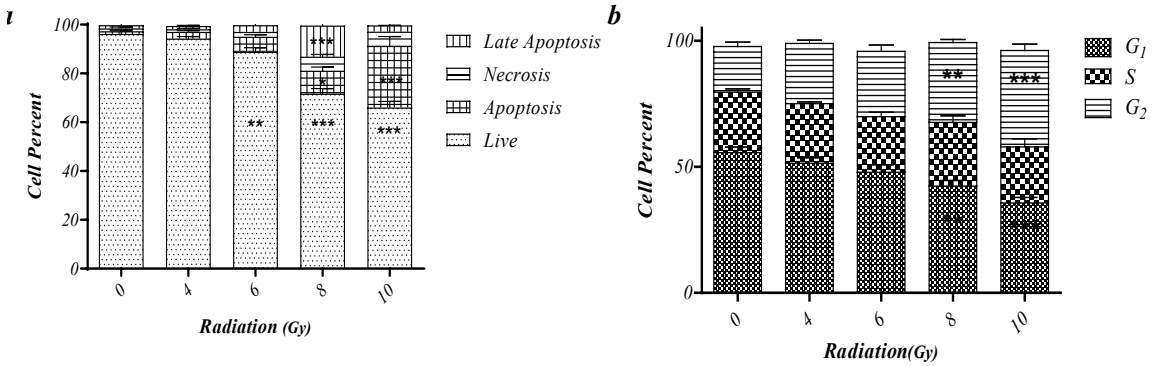

Figure 3. Quantitative Comparison of Apoptosis After RAW264.7 Cell Exposed Groups (0 and 10 Gy). Flow cytometry results for the apoptosis assay and cell cycle distribution. **(a)** Dot plot of apoptosis assays by Annexin V and PI method in cells treated with Gy10 gamma radiation after 24 hours. Q4; live cells, Q3; early apoptosis cells, Q2; late apoptosis cells, Q1; necrosis cells. **(b)** Flow cytometry apoptosis histogram of a different treatment. **(c)** Gy10 gamma radiation reduces live cell percent and arrests cells in the G2/M phase. **(d)** Flow cytometry cell cycle histogram of a different treatment. Radiation reduces live cell percent and increases in apoptotic population. The obtained results are expressed as mean ± SD of at least three replicates (* $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$)

determined by cytokine detection ELISA kits. As shown in Figures 7a, 7b and 7c, the treatment of RAW264.7 macrophage cells with LPS, 4, 6, 8, and 10 Gy irradiation significantly increased the secretion of IL-1β, IL-6, and TNF-α to the cell culture media. The induction effect of the cytokines by 10 Gy radiation was significantly greater than other groups. These findings strongly suggest that radiation is able to modulate inflammatory action by inducing the release of pro-inflammatory cytokines (Figure 7a-c).

NO and PGE2 Production

Under LPS treatment as a model agent for the elicitation of the inflammatory pathway in macrophage cells, gamma radiation (4, 6, 8, and 10 Gy) caused the release of nitrite oxide as an inflammation signal mediator from the treated cells in the culture media, which was determined using the Griess reaction method assay. Gamma radiation significantly induced NO production in a dose-dependent manner (Figure 8a), with an increase in the dose of gamma irradiation leading to an elevation of NO release to the media.

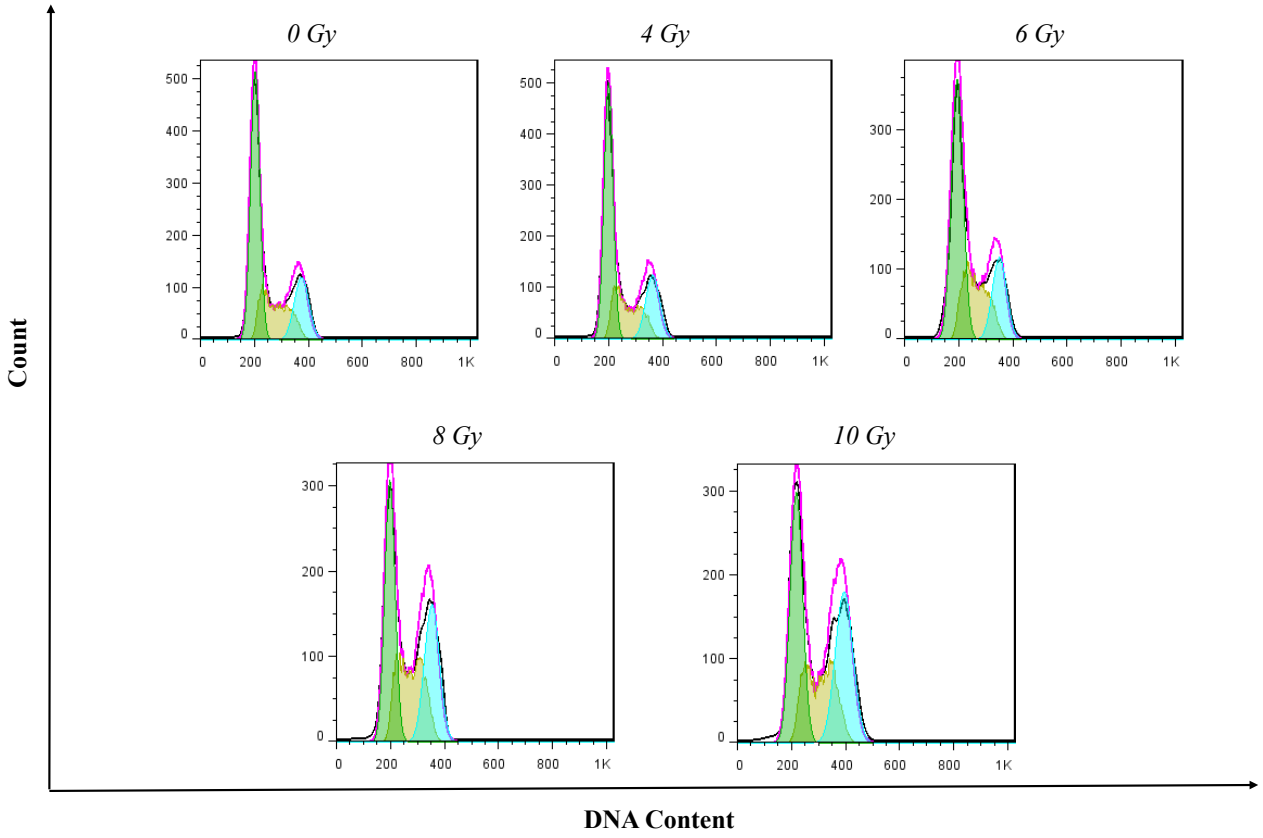

Figure 4. Flow Cytometry Cell Cycle Histogram of Different Treatment

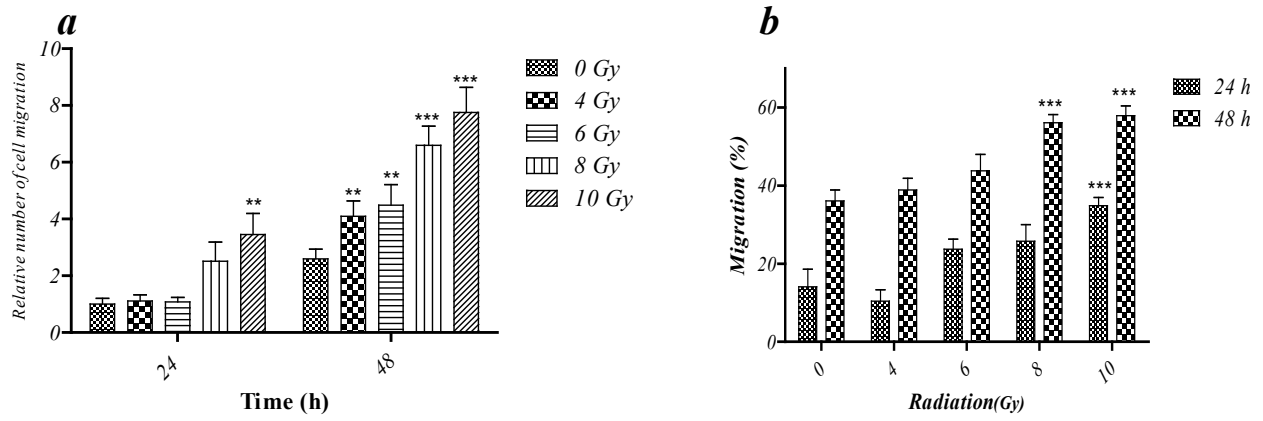

Figure 5. Migration Assay of Macrophage Cells by Gamma Radiation. **(a)** Relative to the control, the fold increases of migrating cells are shown as the means of the cell numbers in fifteen randomly selected visions. **(b)** Migration graph of different treated groups. **(c)** The obtained results are expressed as mean ± SD of at least three replicates (** $P \leq 0.01$, *** $P \leq 0.001$)

Contrastingly, another inflammation signal mediator, PGE2, was assayed using a PGE2 detection ELISA kit, which showed that gamma radiation significantly enhanced PGE2 production (Figure 8b). Therefore, the results demonstrated a positive linear correlation between the dose of irradiation and the release of biochemical inflammation mediators.

Gamma Radiation Increases Apoptotic Genes Expression

The results showed that the exposure of macrophages to 6, 8, and 10 Gy gamma radiation caused an increased expression of BAX and p53 as pro-apoptotic genes, while the expression of Bcl-2 (anti-apoptotic) was found to be down-regulated at 10 Gy (Figure 9a). The results also indicated that Bcl-2 expression was not affected by radiation, showing that a low dose (4 Gy) had no significant effect.

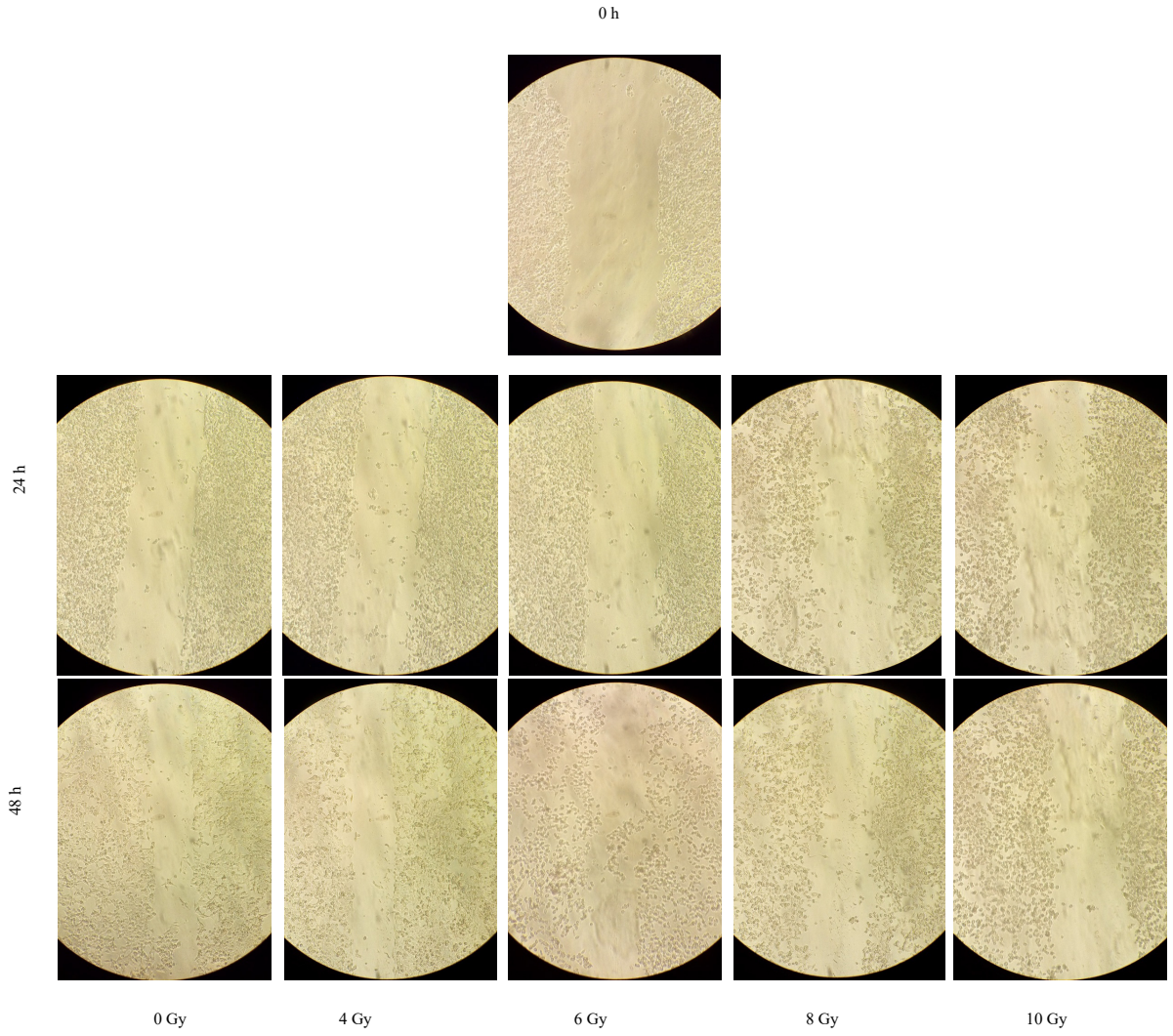

Figure 6. Scratching Assay in Different Times and Radiation Doses

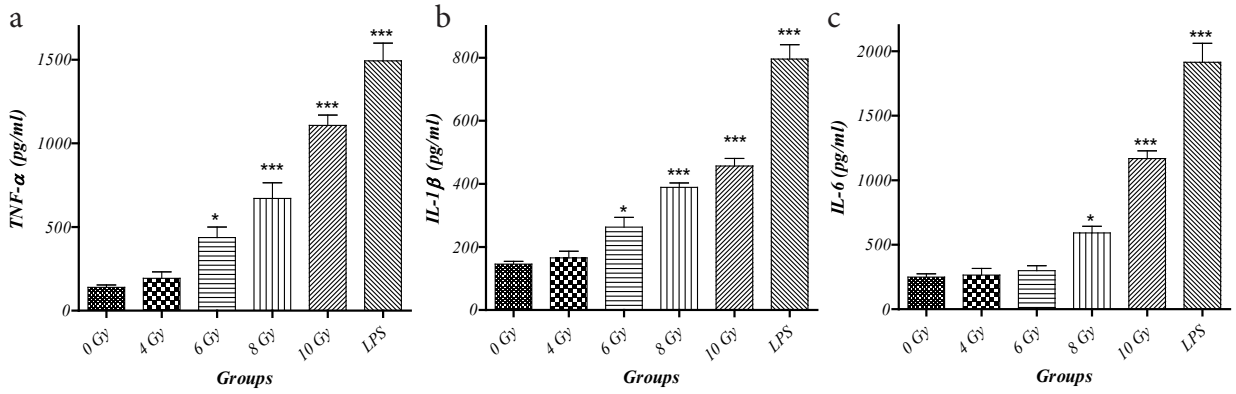

Figure 7. Effect of LPS and Gamma Radiation (4, 6, 8 and 10 Gy) on Pro-inflammatory Cytokine Production in RAW264.7 Macrophage Cells. (**a**) TNF-α, (**b**) IL-1β and (**c**) IL-6. The obtained results are expressed as mean ± SD of at least three replicates (* $P \leq 0.05$, *** $P \leq 0.001$)

Gamma Radiation Up-regulates Inflammatory Genes

The expression of TNFα, IL-6, IL-1β, and iNOS genes, according to qPCR analysis results, showed a significant increase in both radiation-exposed groups and the LPS model group. The expression of inflammatory genes was slightly higher in the 4 Gy group than in the control, but there was no statistical difference (Figure 9b).

Discussion

The application of ionizing radiation in cancer therapy

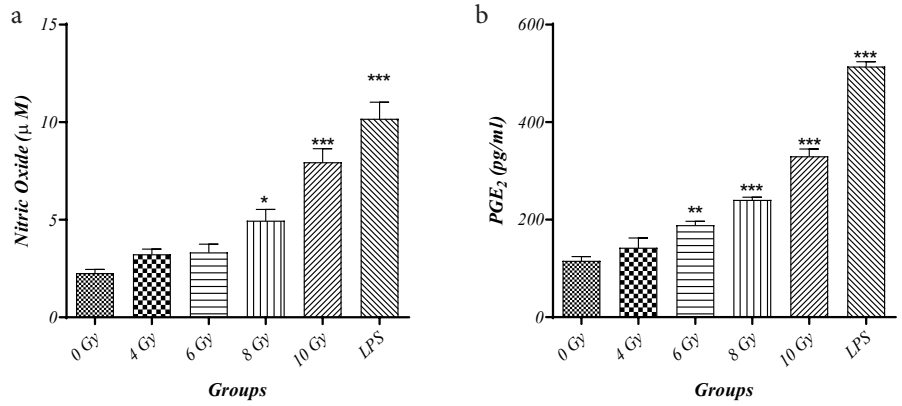

Figure 8. Induced NO Production by Gamma Radiation in a Dose-Dependent Manner. (**a**) NO production of LPS and gamma radiation (4, 6, 8 and 10 Gy) groups was analyzed by a Griess reagent assay. (**b**) PGE2 production of LPS and gamma radiation (4, 6, 8 and 10 Gy) groups was analyzed using a PGE2 detection ELISA kit. The obtained results are expressed as mean ± SD of at least three replicates (* $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$)

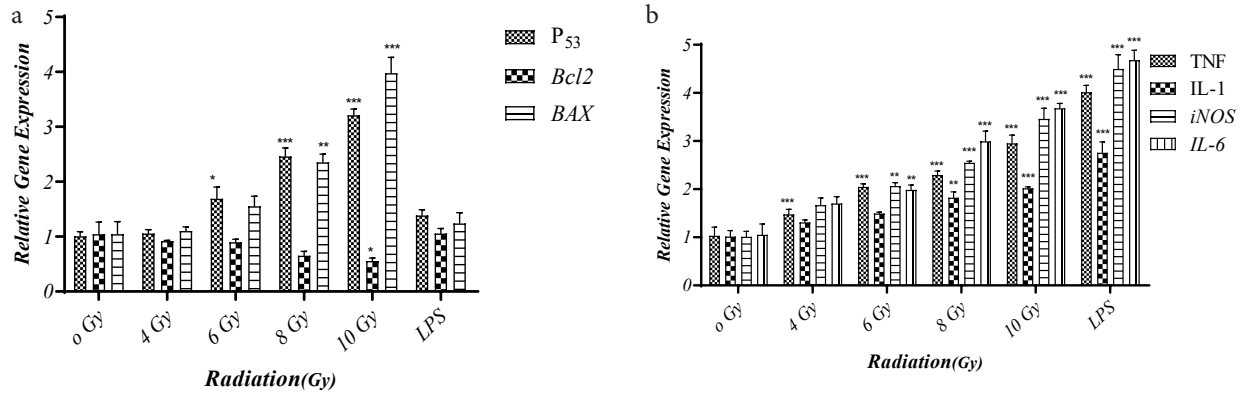

Figure 9. Real-Time Quantitative Analysis and Effect of Gamma Radiation on Pro-inflammatory Cytokines. Real-time quantitative analysis of the fold change of apoptotic (a) and inflammatory (b) genes after irradiation (relative to the control). The obtained results are expressed as mean ± SD of at least three replicates (* $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$)

has improved over the last few years. It has been shown that low doses of radiation are more effective than daily conventional doses of 1-2 Gy in cancer therapy. Several studies have illustrated that ionizing radiation could cause diverse effects on cell biology. Therefore, it seems to be a long way to have a clear perspective on the dose-dependent effects of radiation on biological systems. Low-dose ionizing radiation could improve physiological functions. However, all of these studies were performed under certain exposure conditions, cell lines, or animal models.[21] Direct or indirect exposure of cells to ionizing radiation induces various biological pathway alterations, such as frequent enhancement of micronucleation, apoptosis, mutations, DNA strand breaks, alteration of regulatory protein activity and enzymes, oncogenic transformation, and reduction of clonogenic efficiency.[22] Up to 10 Gy whole-body gamma radiation in animal models can cause an acute induction of the inflammatory system and a cytokine expression burst.[23] The systemic effect of low-dose radiation on the immune system is still obscure. Some studies report that low-dose radiation induces the immune system, while others show a suppressive effect on the immune system.[14] In fact, further studies are needed to clarify the molecular events included in this progress. A low dose of gamma radiation has various effects on living cells, including the enhancement of cell survival, oxidative stress resistance, improvement of immune system function, and stimulation of cell growth. On the other hand, studies show that gamma radiation can cause suppression or modulation of immune system mediators, so it could be assigned as a valuable therapeutic method in autoimmune or chronic inflammatory diseases. As reported, exposure to 0.5 Gy gamma radiation promotes natural killer (NK) cell activity, elevates glutathione levels, and prevents tumor growth factors. It suppresses pro-inflammatory cytokines from peritoneal macrophages and demonstrates strong potential anti-inflammatory effects.[17] 0.5 Gy gamma radiation upregulates MKP-1, activates P38 MAPK, and suppresses TNF-α in mouse macrophage cell lines.[17] Interestingly, recent studies have revealed that low-dose gamma irradiation in collagen-induced arthritis decreases the antibody and pro-inflammatory cytokine release, increases the induction of regulatory T cell count, and thereby causes the attenuation

of disease symptoms.[24] Besides, another study showed that in rheumatoid arthritis, human T lymphocytes were resistant to apoptosis induced by 0.5 Gy gamma radiation. It found that the apoptosis gene expression had a different pattern in the rheumatoid arthritis patients.[25] In this study, doses of 6, 8, and 10 Gy of gamma radiation caused an elevation of apoptotic cells and a decrease of alive cells (Figure 3a). In radiation-induced senescent models, BM-MSCs in vitro increased their expression of IL-6 with reduced immunosuppressive potential. The findings were confirmed in further experiments in vivo when murine MSCs after irradiation also lost their protective immunosuppressive function.[26, 27]

However, the risk of damage to neighboring normal tissues still limits the radiation dose that can be used. In this regard, the potential of the cerulenin synthetic analog C75, a fatty acid synthase inhibitor, and of 17-N-allylamino-17-demethoxy geldanamycin (17AAG), a geldanamycin derivative, to sensitize PCa cells grown in 3D culture to ionizing radiation has been investigated, demonstrating a significant improvement of targeted radiotherapy.[28-30]

According to previous studies, low doses of gamma radiation (less than 50 mGy) have been known to enhance cell mechanisms such as DNA repair and apoptosis reduction, leading to an elevation of cell protective efficiency.[14] Our data have confirmed that the low dose (4 Gy) group did not cause significant apoptosis, while there was a considerable enhancement as radiation increased, especially in high doses such as 8 and 10 Gy. Previous studies have shown that there is a positive correlation between Fas gene expression and gamma irradiation (4 and 10 Gy) in malignant lymphoma cells.[21]

Ionizing radiation induces signaling pathways, such as P53 and Ataxia telangiectasia mutated kinase (ATM), which are involved in DNA damage repair.[17] Although the pre-apoptotic protein (BAX) gene expression was down-regulated, the anti-apoptotic protein (Bcl2) gene was upregulated under low doses of gamma ionization.[31] Radiation can activate cell cycle checkpoints, which arrest the cells at G1/S, S, and G2/M stages.[21] The G2/M checkpoint is the final step before the cells enter mitosis. It has been reported that this checkpoint can be activated as a response to DNA damage. In this study, it was found that in all treated groups, arrested cells in the G2/M phase increased and were remarkable at 8 and 10 Gy treated groups (Figure 2b). It seems that the increasing gamma radiation enhances DNA damage and subsequently elevates the number of cells arrested in G2/M. This is aligned with previous results that reported sub-low dose radiation (2.5 Gy) caused cell accumulation in the G2 phase.

In these radiation doses, cells can enter mitosis or apoptosis progress after the treatment.[32] It is important to note that ionizing radiation can also lead to secondary malignancy when used for cancer therapy, especially when some cells remain alive and can turn into metastatic or invasive forms. It has been reported that ionizing radiation can elevate the motile or metastatic form of cancer cells in vitro by hyper-activating the TGF-β signaling pathway in human carcinoma cells.[33]

Low-dose ionizing radiation can suppress mast cell migration and subsequently inhibit PI3K and Btk signaling pathways through the knockdown of Nr4a2. It was found that a low dose of radiation could down-regulate chemotactic cytokine MCP-1 (monocyte chemoattractant protein 1) gene expression mediated by Nr4a2 in mast cells and suppress cell migration.[34]

This study revealed that macrophage exposure to gamma radiation reduced the anti-inflammatory mediator's profile. It could suggest that pro-inflammatory macrophages may contribute to the effect of local radiotherapy. Pro-inflammatory macrophages produce cytokines such as IL-1B, IL-6, and TNF-α and express genes such as CD80, CD86, HLA-DR, CCR7, and NFKβ involved in cell cycle regulation. On the other hand, anti-inflammatory macrophages induce anti-inflammatory cytokines such as IL-10 and TGF-β and protein expression of CD163 and MRC.[35] The study illustrated that ionizing radiation can regulate macrophages and change their phenotype to the pro-inflammatory form. Therefore, it could be suggested that the regulation of inflammatory responses under gamma radiation exposure is derived by promoting the production and release of pro-inflammatory cytokines (Figure 7a-c).

According to previous studies, gamma irradiation can enhance NO production in RAW264.7 cells, leading to DNA damage through the induction of the nuclear factor pathway (NF)- κβ.[36] A recent study also showed that NO and PGE2 biosynthesis are enhanced in a dose-dependent manner after gamma radiation treatment (Figure 8b). As it is clear, the iNOS gene was upregulated in both radiation and LPS groups. The determination of genes stimulated by gamma radiation in Raw264.7 cells shows that the gene expression of pro-inflammatory cytokines TNFα, IL-6, IL-1β, and iNOS substantially increased in radiation-treated groups, similar to the LPS-induced model. The expression of pro-inflammatory genes was slightly higher in the 4 Gy group compared to the control group, but there was no statistical difference (Figure 9b).

Finally, the current study suggests that Raw264.7 cells can serve as key markers for inflammatory diseases exposed to gamma radiation (0, 4, 6, 8, 10 Gy). Higher doses of radiation damaged the DNA of the cells, causing them to be arrested at the G2/M phase. If the cells could not repair the damage (which is common at higher doses), apoptosis genes such as P53 and BAX were upregulated. In fact, there is a bidirectional effect between the P53 and BAX genes, where P53 regulates BAX, and BAX is involved in the P53 apoptosis mechanism. Therefore, an

increased expression of both allows the cells with DNA damage to enter the apoptotic pathway. Conversely, Bcl2 (which is a type of anti-apoptotic gene) is down-regulated and aligned with this process. Moreover, pro-inflammatory cytokines (IL1B, IL6, and TNF-α), NO, and PGE2 production are enhanced during DNA damage and apoptosis steps. NO burst is closely linked to excessive activation or production of different inflammatory cytokines like TNF-α, IL6, and IL1B.[36] Although high doses of radiation caused most cells to enter the apoptosis phase and the number of viable cells reduced, the current study revealed that these results were not considerable at 4 Gy radiation. Interestingly, high gamma radiation could increase cell motility and migration, and this effect cannot be neglected, especially when used in cancer therapy, because it may turn cells into a metastatic form.

Conclusion

In recent times, gamma radiation has been extensively used as a therapeutic choice in medicine. However, the therapeutic use of low doses of gamma radiation, which could be beneficial in cancer therapy and suppression/modulation of immune responses in chronic inflammatory disease, has different doubtful aspects that should be carefully cleared. The results of this study showed that 4 Gy gamma radiation had no destructive effect on the macrophage cell line. However, higher doses led to the progression of apoptosis in the cells, driven by irreversible damage at the cellular and molecular levels.

Authors' Contribution

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Competing Interests

There is not any conflict of interest.

Ethical Approval

This research was approved by the research ethics committee of Shahid Beheshti University of Medical Sciences (IR.SBMU.RETECH.REC.1401.224).

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