

## INF/IL-4 increases after the low doses of gamma radiation in BALB/c spleen lymphocytes

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### ABSTRACT

**Introduction:** The effects of the low dose of ionizing radiation are not thoroughly evident due to an unavoidable increase of occupational exposure and the widespread application of ionizing radiation in medical and industrial fields. The aim of this study was to investigate immune system responses following the low doses of ionizing radiation.

**Material and Methods:** BALB/c mice were exposed to Whole Body Irradiation of 20, 50, and 100 mGy through a <sup>60</sup>Co source. Lymphocytes extraction were operated 24 h after irradiation. Afterwards, gene expression analysis was performed with relative quantitative Real-Time polymerase chain reaction to IL-4, IFN- $\gamma$ , and TGF- $\beta$  expression levels. Moreover, IFN- $\gamma$ /IL-4 ratio was computed, and the independent sample t-test was performed for the statistical analysis.

**Results:** Whereas IL-4, IFN- $\gamma$ , and TGF- $\beta$  expression levels decrease after the radiation of the low doses of gamma rays, the IFN- $\gamma$ /IL-4 ratio increased significantly after irradiation of 20 mGy (P-Value<0.05). However, this ratio did not vary following the gamma irradiation of 50 and 100 mGy.

**Conclusion:** The positive effects of the low dose of ionizing radiation can be observed through significant alterations in gene expression and the activation of protection mechanisms. This process was caused by the modulation of cellular immune responses after the exposure to 20 mGy. Although the irradiation of higher doses (50 and 100 mGy) induced alterations in the IFN- $\gamma$ /IL-4 ratio was insignificant. These findings did not confirm the linear no-threshold model theory, and demonstrated that the low dose of ionizing radiation could be the cause of the hormesis phenomenon.

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### Introduction

Nowadays, there has been a rapid increase in the utilization of ionizing radiation in medical and industrial fields. Despite the widespread use of ionizing radiation, its detrimental biological effects cannot be ignored. As a result, it is important to consider the harmful effects of ionizing radiation, especially in low doses.

To estimate possible harmful effects induced by the low doses of ionizing radiation (LDIR-below 100 mGy), linear non-threshold (LNT) model extrapolates the obtained data from the higher doses of ionizing radiation. According to the LNT model, it is impossible to eliminate the risks of ionizing radiation. In most countries, recommendations provided by the International Commission on Radiological Protection (ICRP) are widely used to establish radiation protection guidelines. These recommendations are mostly based on the LNT model [1-3]. Although the

LNT model considers low dose radiation dangerous, numerous observations suggested the benefits of low-dose exposure, such as adaptive response, radiation hormesis, prolongation of life span, immunologic enhancement, treatment or suppression of disease, and reduced chromosomal aberrations [4-7]. These issues are recognized as the positive effects of LDIR on organisms.

The effects of LDIR on the immune system are still controversial, and the results of investigations show repugnant consequences [8]. While several reports pointed out the immune suppression effects of LDIR [9], other investigations demonstrated that LDIR leads to immune stimulation [10]. The immune suppression induced by low dose radiation with a combination of different mechanisms could limit immune recuperation [11], increase life span [12, 13], and stimulate the immune system [14, 15]. However, ICRP

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Publication 103 reported a specified dose limit of 20 mSv per year for occupational exposure averaged over 5 years, maximum of 50 mSv in a single year, and 100 mSv per 5 years. However, there is no evidence on the effects of these limits on the immune system.

Furthermore, previous investigations demonstrated that occupational exposure could be carcinogenic [16-19]. Since the induction of cancer in low doses of ionizing radiation may be due to the suppression of immune system, it seems that the study of the effects of low doses on the immune system would have beneficial results for those whose jobs involved radiation. The aim of this study was then to investigate the effects of delineated dose limits of ionizing radiation proposed by the ICRP for radiation-exposed workers at IL-4, IFN- $\gamma$ , and TGF- $\beta$  expression levels and immune system responses in the spleen lymphocytes of BALB/c mice.

## Materials and Methods

### Animals

In the present experimental study, male BALB/c mice were purchased from the Pasteur Institute, Karaj, Iran. The mice were within the age range of 5-6 weeks and were kept under standard vivarium conditions ( $21\pm 2^\circ\text{C}$  room temperature, 65-70% humidity, natural light conditions, and standard mouse diet).

The animals were divided into four groups (8 mice in each group). The four groups were exposed to (a) 0 mGy (control), (b) 20 mGy, (c) 50 mGy and (d) 100 mGy. The age range of mice was 7-8 weeks at the time of irradiation.

### Gamma irradiation

A 0.6 cm<sup>3</sup> Farmer ionization chamber and a Farmer electrometer model 2581 were utilized to measure the absorbed dose in a water phantom, with the depth of 5 cm, SSD of 95 cm, and field size of 7 $\times$ 18 cm<sup>2</sup> (same as irradiation). Absorbed dose incurred from Whole Body Irradiation (WBI) was calculated at a depth of 1.5 cm (approximately the mid-depth of the mouse's body). All four groups of mice, housed in a plastic cage, were exposed to  $\gamma$ -rays of a Co-60 source without any anesthesia. The dose rate of the source at the time of irradiation was 31.64 mGy/min (without using shielding), an average of 3 times measurement.

### Preparation of White blood cells (WBCs)

The animals were euthanized 24 h after irradiation by cervical dislocation (without anesthesia), and their spleens were removed in an aseptic situation. The splenocytes were emitted by the injection of 10 ml RPMI 1640 (GIPCO) into the spleens, and the obtained solution was centrifuged. After the removal of the supernatant, the RBCs of splenocytes were lysed by means of washing with 13 ml of Ammonium chloride (containing (NH<sub>4</sub>Cl), sodium bicarbonate and EDTA). The remaining white blood cells were washed twice with 10 ml RPMI.

### RNA extraction

TriPure reagent (produced by Roche Applied Science, Germany) was used for RNA isolation. One ml TriPure reagent was added to each pellets containing (5-10)  $\times 10^6$  cells and each sample was incubated for 1-5 min at room temperature. Afterwards, 200  $\mu$ l Chloroform (Merck, Darmstadt, Germany) was added to each sample, and diametrical was shaken manually for about 15 sec. After the incubation of all samples at room temperature for 15 min, the mixtures were centrifuged at 15000 g and 4  $^\circ\text{C}$  for 15 min to separate the solutions into 3 phases. The colorless upper phase containing RNA was derived after adding 500  $\mu$ l Isopropanol (Merck, Darmstadt, Germany), and the sample was shaken for about 15 sec. After the incubation of the samples for 15 min at room temperature and centrifugation for 15 min at 15000 g and 4 $^\circ\text{C}$ , the RNA samples were washed with 96% Ethanol (Merck, Darmstadt, Germany), and dried in the blast. The RNA was diluted with 30  $\mu$ l DiEthylPyroCarbonate (DEPC)-treated RNase-free water and the mixture was then resuspended.

### cDNA synthesis

For cDNA synthesis, RevertAid<sup>TM</sup> First Standard cDNA Synthesis Kit (Fermentas) was employed and cDNA was synthesized according to the manufacturer's recommendations. In the thermocycler, the conditions of reactions were 65 $^\circ\text{C}$  for 10 min, 42 $^\circ\text{C}$  for 60 min, and 70 $^\circ\text{C}$  for 5 min. Polymerase chain reaction (PCR) was used for cDNA sample strengthening. For PCR, GAPDH primers were used and a lucid 496 bp was observed for all the PCR products after loading on 1% agarose gel. All of the samples were stored at -70 $^\circ\text{C}$  for future use.

### Gene expression (Real-Time PCR)

The SYBR<sup>®</sup> Premix Ex Taq<sup>TM</sup> (Takara, Japan) was employed to analyze the gene expression of the investigated genes (i.e., IL-4, IFN- $\gamma$ , and TGF- $\beta$ ). The qPCR was accomplished with a StepOne<sup>TM</sup> Real-Time PCR System and in a total volume of 15  $\mu$ l, containing 7.5  $\mu$ l of SYBR<sup>®</sup> Premix Ex Taq<sup>TM</sup>, 0.3  $\mu$ l of ROX<sup>TM</sup> Reference Dye II, 300 nM of forward and reverse primers, 1.5  $\mu$ l of cDNA (density of 0.1), and 5.1  $\mu$ l of dH<sub>2</sub>O as duplicates. The optimized thermal cycling conditions in the qPCR were set in the following conditions: at first, for 10 min in 95 $^\circ\text{C}$  (denaturation), thereafter 40 cycles (2 steps) for 15 sec at 95 $^\circ\text{C}$  (denaturation), and 60 $^\circ\text{C}$  for 30 sec (annealing) for IL-4 and IFN- $\gamma$  expression analysis. For the TGF- $\beta$  gene, the optimized conditions were slightly different, meaning that 95 $^\circ\text{C}$  for 10 min (denaturation), followed by 40 cycles at 95 $^\circ\text{C}$  for 15 sec (denaturation), 55 $^\circ\text{C}$  for 60 sec (annealing), and finally 72 $^\circ\text{C}$  for 60 sec (primer extension). Table 1 shows the sequence of primers in Real-Time PCR.

Table 1. Sequence of Primers Used for Real-Time polymerase chain reaction

Name (product length)	Sequence
GAPDH (98 bp)	Forward: AACTCCCATTCTTACCTTTG Reverse: CTGTAGCCATATTCATTGTCATACCAG
IL-4 (147 bp)	Forward: ACCACAGAGAGTGAGCTCGTCT Reverse: TGAATCCAGGCATCGAAAAG
IFN- $\gamma$ (127 bp)	Forward: GAACTGGCAAAGGATGGTGA Reverse: GTGTGATTCAATGACGCTTATGTT
TGF- $\beta$ (88 bp)	Forward: CGGACTACTACTATGCTAAAGA Reverse: CTGTGTGAGATGTCTTTG

### Data analysis and statistics

The quantitative relative method in Step One software (2.1, Applied Biosystems) was used to analyze expression patterns. The normalized quantity mean (NQM) and the relative quantity (RQ) were obtained by the normalization of the target genes quantity into the quantity of mouse GAPDH (as housekeeping gene) and division of NQM of the irradiated samples into NQM of the control samples, respectively. In the last step, Log base 2 of the RQs were obtained for statistical analysis and drawing plots.

The obtained data were analyzed using SPSS software (version 20). The independent sample t-test was performed to analyze gene expression changes and to compare gene expression levels. P-value less than 0.05 was considered statistically significant.

## Results

The present study was an investigation of the effects of LDIR on IL-4, IFN- $\gamma$ , and TGF- $\beta$  genes expression levels in the lymphocytes of the BALB/c mice's spleens through total body irradiation. In this regard, Real-Time PCR method was utilized. Furthermore, the IFN- $\gamma$ /IL-4 ratio was computed for each sample. The calculation of the number of cells indicated that the low doses of ionizing radiation caused a significant reduction in the numbers of lymphocyte cells (Figure 1).

The obtained results of the study revealed that in the groups irradiated by 20 and 50 mGy of ionizing radiation, there was a significant reduction of IL-4 gene expression 24 h after irradiation. This down-regulation was insignificant for the group, which received 100 mGy of gamma rays (Figure 2.A). The results also showed that a significant down-regulation of IFN- $\gamma$  gene expression was induced as a result of 20 mGy after 24 h. However, the induced changes by 50 and 100 mGy were insignificant (Figure 2.B). Moreover, the expression of TGF- $\beta$  was found to be down-regulated at all doses (20, 50, and 100 mGy), 24 h after irradiation (Figure 2. C).

The IFN- $\gamma$ /IL-4 ratio was calculated and investigated as an indicator of the Th1/Th2 response ratio. The quantity of the induced changes of Th1/Th2 ratio indicated an extent of immune responses shifting towards cellular or humoral immunity. The LDIR caused a significant increase of IFN- $\gamma$ /IL-4 ratio following a dose of 20 mGy; however, this ratio remained unchanged following the doses of 50 and 100 mGy (Figure 2 D).

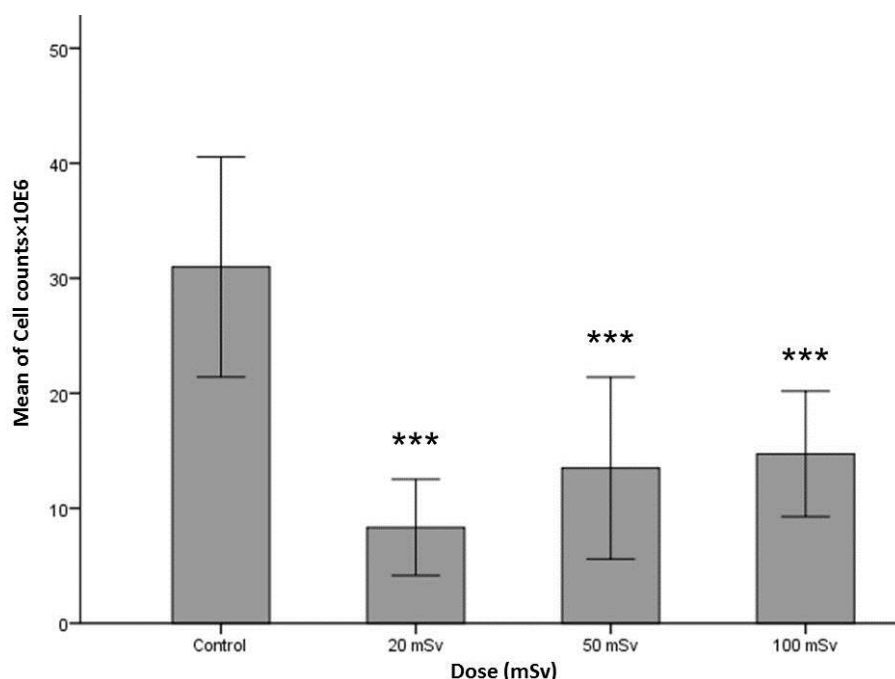


Figure 1. Levels of the numbers of counted cells in the groups by control, 20, 50, and 100 mGy gamma irradiation. Error bars represent standard deviations. Significance of changes is indicated by \* (P-value < 0.05), \*\* (P-value<0.01) or \*\*\* (P-value<0.001).

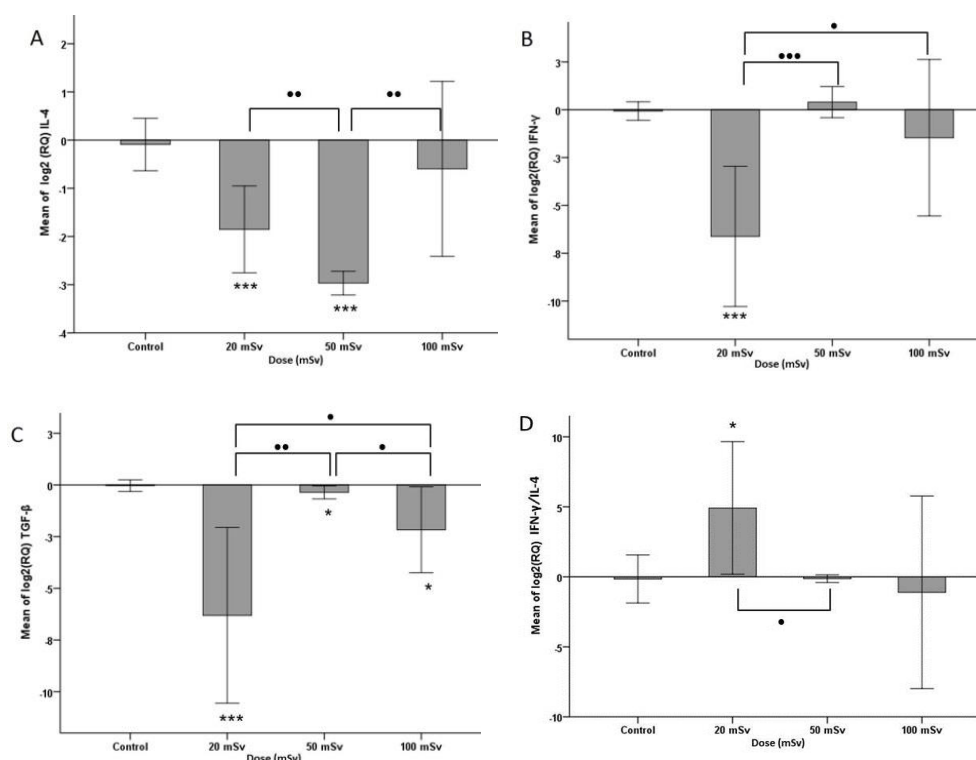


Figure 2. Alterations at the levels of gene expression of lymphocytes in the BALB/c mice after Gamma irradiation ( $^{60}\text{Co}$ ) for IL-4 (A), IFN- $\gamma$  (B), TGF- $\beta$  (C), and the IFN- $\gamma$ /IL-4 Ratio (D). The error bars show standard deviations for each group. Significance of induced changes between the irradiated groups and the control group is signified by \* (p-value < 0.05) or \*\*\* (p-value < 0.001). The significance of differences among the irradiated groups is shown by • (p-value < 0.05), •• (p-value < 0.01) or ••• (p-value < 0.001).

## Discussion

Since the effects of LDIR on the immune system are not quite obvious, and the immune system function could affect different parts of the body (e.g., immune system suppression could trepan to cancer formation), the aim of the current study was to investigate the eliminated doses proposed by ICRP for radiation-exposed workers.

The obtained results of the current study were indicative of a reduction in the number of counted lymphocytes after irradiation of 20, 50, and 100 mGy, which could induce apoptosis induction. Moreover, it is important to mention that some other factors, such as inter-individual variability between the mice and the quantity of cell extraction, could affect the number of counted cells as well as irradiation dose.

Furthermore, the obtained data showed that the low doses of ionizing radiation could reduce the IL-4 expression level 24 h after irradiation. Likewise, Bogdändi et al. reported similar results in low and high doses [20]. Contrary to the results of the present study, Shin (2010) observed an increased level of IL-4 expression after the 200 mGy irradiation to the lymphocytes of the spleen and peripheral blood cells [21] due to a lower dose rate (0.7 mGy/hr). In other words, the high and low doses of ionizing radiation can lead to a diverse range of responses.

Exposure to 20 mGy dose of gamma irradiation induced a down-regulation in IFN- $\gamma$  expression 24 h after irradiation, while no significant alteration in the

levels of IFN- $\gamma$  expression was noticed in the groups exposed to 50 and 100 mGy doses. Similar to the findings of the current study, previous studies demonstrated a reduction in IFN- $\gamma$  expression patterns in the radiation-exposed workers [22] and after 10 mGy irradiation [20]. One of the Th1/Th2 response indicators is IFN- $\gamma$ /IL-4 ratio and the cytokines, which are synthesized by Th1 (type-1 T helper) and Th2 (type-2 T helper) cells. This issue can induce cellular immunity and humoral immunity, respectively [23, 24]. Since IL-4 and IFN- $\gamma$  are respectively indicators of TH1 and TH-2 responses, the down-regulation of these cytokines could be caused by immune suppression.

The results of this study were consistent with Bogdändi et al., indicating that irradiation with low doses (20, 50, and 100 mGy) could significantly reduce the expression level of TGF- $\beta$  gene after 24 h [20]. As mentioned earlier, TGF- $\beta$  is one of the cytokines of Treg cells and inhibits Th1 and Th2 activations. Therefore, the prerequisite to down-regulation in the IL-4 and IFN- $\gamma$  expressions after immune system suppression is the up-regulation of TGF- $\beta$  expression. Furthermore, it is envisaged that the effects of irradiation of 50 and 100 mGy on the immune system suppression are more than irradiation of 20 mGy. However, maximal induced alterations were observed in the group exposed to 20 mGy. Accordingly, changes at the level of gene expression could not be directly attributed to the immune system. The differences between the results of the present study and those in the



literature showed that LDR led to an up-regulation in TGFB as a result of the different doses (300 and 500 mGy).

The IFN- $\gamma$ /IL-4 ratio increased significantly following the irradiation of 20 mGy 24 h after exposure, while 50 and 100 mGy irradiation did not change this ratio, compared to the control group. However, another study reported IFN- $\gamma$ /IL-4 ratio reduction after a dose of 200 mGy [21]. The difference in the obtained results can be due to the irradiation of a higher dose, compared to the present study.

Accordingly, the significant increase of this ratio in the group irradiated to 20 mGy could be an indicator through which immune responses shifted to Th1 responses, and thereby to cellular immunity. Moreover, a reduction in the levels of gene expressions was observed in all of the investigated genes.

In recent years, several studies have confirmed the adaptive response via DNA repair and deletion of damaged cells through apoptotic mechanisms or immune responses [25]. In previous studies, the results demonstrated that the low doses of Gamma irradiation (20, 50 and 100 mGy) reduce apoptosis in human peripheral blood lymphocytes 4, 24, 48, 72, and 168 h after irradiation [26]. The results of another study indicated the significant reduction of DNA damage in thymus cells of mice following the exposure of 50 and 75 mGy after 24 h [27]. Furthermore, the reduced apoptosis in splenocytes was observed 4 h after the irradiation of 10 mGy, and relative cell numbers of such different splenocyte subpopulations were significantly increased 24 h after 10 mGy of gamma irradiation [20]. Based on the results of previous studies, the low doses of ionizing radiation (especially doses of less than 50 mGy) could activate the protection mechanisms in cells, such as an increase in DNA repair and numbers of cell populations as well as a reduction in apoptosis [28, 29]. Based on the aforementioned literature and the obtained results of the current study (i.e., no immune system activation), it can be hypothesized that the eventual effects of irradiation with 20 mGy might be the activation of DNA damage repair. Due to this significant reduction in the expression levels of all the investigated genes (IL-4, IFN- $\gamma$ , and TGF- $\beta$ ) in this group, significant repair and protection mechanisms were activated after irradiation.

Since the cells are in a special phase (e.g., mitosis or mechanisms of protection), the levels of unnecessary gene expression are reduced. Accordingly, after the irradiation of very low doses and activation of protection mechanisms, the levels of immune system genes (as IL-4, IFN- $\gamma$ , and TGF- $\beta$ ) that are unnecessary genes will be reduced. In other words, the observed down-regulation of the IL-4, IFN- $\gamma$  and TGF- $\beta$  expressions after exposure to 20 mGy could be the result of cell entry into protection mechanisms, and the reason for these alterations was not immune system suppression.

However, the induced changes in the levels of investigated gene expressions could be an outcome of

the apoptosis incidence. The reason resulted from the insignificant alterations of the IFN- $\gamma$ /IL-4 ratio after the exposure to 50 and 100 mGy, confirmed by the current study and the one conducted by Bogdändi et.al. (2010; followed a similar research method to the present study).

According to the results of this study, it can be concluded that the immune system cannot be activated by 20 mGy. On the other hand, based on other studies, it can be hypothesized that the protective effect of LDR is due to mechanisms other than the activation of the immune system, including DNA repair activation and lower apoptosis.

## Conclusion

The reduction in the genes of the immune system and the alteration of the IFN- $\gamma$ /IL-4 ratio with a shift of immune responses towards cellular immunity after low dose irradiation. This means that after irradiation with low doses of ionizing radiation immune system suppression does not happen. As a result, carcinogenesis following LDIR irradiation is not due to immune system injury. These results can be essential to the radiation-exposed workers and patients who are affected by autoimmune diseases.

Furthermore, the LNT model is considered the base for all radiation protection recommendations of ICRP, IAEA (International Atomic Energy Agency), and other subordinate organizations that are followed in many countries. This theory extrapolates the effects of LDIR obtained from a high dose of ionizing radiation (atomic bomb survivor investigations) although it was criticized in recent years for some concepts, such as hormesis. Since the induced responses by the low dose of ionizing radiation have not been predicted by the LNT theory, the other important outcomes of the present study are in contrast with the obtained results of the LNT theory, and confirmation of the hormesis hypothesis by our results.

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