ORIGINAL ARTICLE

The Effects of Vitamin A and Magnesium Sulfate on the Expression of Nox_4 Following Irradiation in Bone Marrow Cells

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Abstract

Purpose: Nicotinamide Adenine Dinucleotide Phosphate (NADPH) oxidase is one of the probable sources of Reactive Oxygen Species generated by ionizing radiation. This study aimed to determine the expressions of Nox_4 and radioprotective effects of magnesium sulfate and vitamin A following whole-body 2 Gy X-ray irradiation.

Materials and Methods: In this research, 6-7 weeks old Naval Medical Research Institute (NMRI) male mice were kept in a standard cage with an appropriate temperature and 12 on 12 off light cycle. Three concentrations of vitamin A and magnesium sulfate were intraperitoneally injected into mice 2 hours before irradiation. The dose rate was 50 cGy/min at Source Skin Distance (SSD) = 100 cm and a field size of 10 × 10cm^2. The mice were anesthetized and sacrificed by cervical dislocation 24 hours after irradiation. Then, the expression of Nox_4 was assessed by Real-Time Polymerase Chain Reaction (PCR).

Results: There were significant differences between the mean of gene expression in groups treated with vitamin A and magnesium sulfate compared to only radiation group ($P < 0.05$).

Conclusion: Based on the results of this study, it is likely that vitamin A and magnesium sulfate neutralize the harmful effects of free radicals due to their antioxidant properties.

Keywords: Radiation-Protective Agents; Magnesium Sulfate; Vitamin A; Gene Expression; Nox_4.

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1. Introduction

People are exposed to Ionizing Radiation (IR), one of the gene mutations, in different ways, such as through diagnostic and radiology therapy, and background radiation including cosmic rays and radiations emitted by radioactive substances in the Earth's crust [1-5]. Exposure to IR can cause many detrimental effects including altering the inflammatory moderators and pro-oxidant enzymes that increase the production of free radicals and energetic molecules, such as Reactive Oxygen Species (ROS) [6].

The main product of Nox_4, one of the seven members of the Nox family (Nox_1, Nox_2, Nox_3, Nox_4, Nox_5, $Duox_1$, and $Duox_2$), is Hydrogen Peroxide (H_2O_2). Nox_4-derived H_2O_2, one of the most oxygen sensors, has a large impact on cell migration, proliferation, and death. Overexpression of Nox_4 has been reported in cancer cells, which engage in metastasis, angiogenesis, and apoptosis.

Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase complex are several proteins that are encoded by the NOX_4 gene. They produce ROS, mainly superoxide by transferring an electron from NADPH to molecular oxygen. At moderate and low levels of ROS, it facilitates the function of the immune system and cellular response. Nonetheless, when the concentration of the free radicals is relatively high, they become poisonous and harm the antioxidant defense system which can result in oxidative stress [7]. Since ROS are highly active, they can interact with various macromolecules, such as lipids, proteins, and Deoxyribonucleic Acid (DNA) [8].

ROS are involved in numerous major reactions, including lipid peroxidation, strand breaks, and base modification in DNA [9]. If the oxidative stress is pretty high to change the vital functions of membrane proteins and enzymes, it causes a reduction in the enzymatic activity, deletion of bases, cross-linking of DNA with other proteins or DNAs, single and double stand(s) breaks, and base change [10]. These modifications result mainly in various biological injuries, like gene mutations, chromosome aberration and cell death [11].

DNA of some exposed cells that have evaded death could have been mutated and caused gene expression and protein alteration, including cross-linking. The ROS-mediated DNA injury can be relatively low that allows cells to somewhat continue their functions and proliferate, but they can eventually develop into an abnormal mass of tissue [12].

Many scientists have sought to discover materials that can decrease deleterious outcomes of radiation on living organisms, especially in radiotherapy [13, 14]. According to the Departments of Defense and Health and Human Services, Medical Countermeasures (MCM) are divided into two parts; preventive agents that refer to radioprotectors and mitigators; and therapeutic materials that can treat radiation injury [15].

Radioprotectors should be used before or at the time of irradiation to reduce the destructive effects of radiation on normal cells [16]. Radioprotective compounds are classified into three groups; thiol syntheses that nullify free radicals produced by radiation; cytokines and growth factors that regulate communications between immune cells and other cells, which lead to change in cellular reaction to radiation; and extracts of herbs and natural antioxidants [17].

Although many attempts have been made to achieve an appropriate countermeasure, amifostine (EthyolR) is the only MCM that has been certified by the United States (US) Food and Drug Administration (FDA) to diminish the deleterious injury of radiation in radiotherapy [18]. Amifostine has some limitations, including its side effects that can cause vomiting, nausea and hypotension [19]. As a result, many recent studies have been carried out on natural substances such as vitamins, magnesium sulfate, and flavonoids because of their lesser toxicity [20-22].

It has been shown that antioxidants, such as magnesium, vitamin E, C, and A can offset ROS generated by radiation [12, 23]. Vitamin A might serve as an efficient radioprotector at high local doses in special cells due to its lipophilicity property [24]. Beta-carotene is one of the most typical precursors of vitamin A. Beta-carotene preserves the cell membranes against lipid peroxidation by reducing the production of trichloromethylperoxyls [25]. Some studies have suggested that magnesium plays an important antioxidant role by scavenging free radicals. Furthermore, magnesium declines the amounts of oxidized free radicals by blocking the production of nicotine amide adenine dinucleotide phosphate oxides [26, 27].

Since there has not yet been recognized an efficient, available, and non-toxic radioprotector, this study was performed to scrutinize the radioprotective effect of

vitamin A and magnesium sulfate on the expression of the Nox_4 gene following 2 Gy of X-ray irradiation.

2. Materials and Methods

2.1. Categorization of Animals

In this research, 6-7 weeks old Naval Medical Research Institute (NMRI) male mice weighing 26 – 28 g, were kept in a standard cage with an appropriate temperature and 12 on 12 off light cycle. Each group consists of 5 mice (Table 1). The mice were treated in accordance with the Guide for the Care and Use of Laboratory Animals (8th edition, National Academies Press); also, The Ethical Committee for medical Research at Tehran University of Medical Science confirmed this research [ethical code IR.TUMS.SPH.REC.1396.4098] [28]. Moreover, the ethical code from the Ethical Committee of Tehran University of Medical Sciences was acquired.

Table 1. The animals were categorized into 11 groups according to the following Table

Group Code	Dose of X Radiation (Gy)	Dose of Drugs (mg/kg)
A	0	5 cc normal saline
B	0	5 cc ethanol (5%)
C	2	0
D	0	200 mg vitamin A
E	0	150 mg mgso4
F	2	100 mg vitamin A
G	2	200 mg vitamin A
H	2	400 mg vitamin A
I	2	75 mg $mgso_4$
J	2	150 mg $mgso_4$
K	2	300 mg $mgso_4$

2.2. Dosages of Drugs

The drugs were Vitamin A (DarouPakhsh Pharmaceutical Co, Tehran, Iran) and magnesium sulfate (Merck, Germany, pa). Three groups of mice were given 3 various concentrations of vitamin A (100, 200, and 400 mg/kg). Also, three concentrations of magnesium sulfate (75, 150 and 300 mg/kg) were injected into three groups of animals. In addition, ethanol (%5), the solvent of vitamin A, was injected into a category of animals to find out the difference between the mean expression of the Nox_4 in ethanol and control group. Two hours after the injection of drugs, mice were situated in standard irradiation cages and irradiated to 2 Gy of X radiation by 10 MV x-ray beams from a linear accelerator (Varian 2100 CD). Also, the dose rate was 50 cGy/min at Source Skin Distance (SSD) = 100 cm and the field size of 10 × 10 cm^2.

2.3. Extraction of Bone Marrow

24 hours after irradiation, the mice were anesthetized and sacrificed by cervical dislocation. Subsequently, both femoral bones were taken away, and the bone marrow of each femur was extracted and poured down into a microtube by 1 cc Fetal Bovine Serum (FBS) from the lower end of the femur. All samples were centrifuged at 2000 rpm for 6 min at 4°C and supernatants were removed; then microtubes were kept at -70 ° C for Ribonucleic Acid (RNA) extraction.

2.4. Gene Expression Method

Generally, gene expression includes three phases; extraction of RNA from bone marrow tissues that was done by RNeasy Mini Kit (Qiagen) and its quality and quantity were assessed using agarose gel (1.5%) and spectrophotometer (Thermo Scientific™ NanoDrop-1000); cDNA synthesis (RT-PCR), 2 micrograms RNA were transformed into cDNA by SuperScript II reverse transcriptase (Invitrogen), and Oligo (dT)15 primer (Roche) that anneal to the poly A of mRNAs; Real-time PCR that requires some agents, such as characteristic primers of Nox_4 (Table 2), LightCycler® FastStart DNA MasterPLUS SYBR Green I, and a LightCycler Real-time machine (Roche). Glyceraldehyde 3-Phosphate Dehydrogenase (GAPDH) gene was used as a housekeeping gene (Table 2).

Table 2. Forward and reverse primers of Nox_4 and GAPDH genes

Primers	Sequence
GAPDH F	5-CCCTTAAGAGGGATGCTGCC-3
GAPDH R	5-TACGGCCAAATCCGTTCACA-3
Nox_4 F	5- TTGCCTGGAAGAACCCAAGT-3
Nox_4 R	5- TCCGCACAATAAAGGCACAA-3

2.5. Statistical Analysis

The Normality of data was carried out by Kolmogorov–Smirnov test in SPSS 20; then to evaluate the differences in mean gene expression of Nox_4 in studied groups, the Kruskal Wallis test and one-way Analysis of Variance (ANOVA) were, respectively, applied for abnormal and normal distributions. If the data distribution was normal, Tukey's Post Hoc Test was used to recognize the differences between various groups ($P < 0.05$).

3. Results

Table 3 shows the Mean ± standard error of the mean (SEM) of the expression of the Nox_4 gene in animals treated with vitamin A, 24 h after irradiation. There was a significant difference between the expression of the Nox_4 gene in group C and group D ($p < 0.05$); there were also significant differences in the expression of the Nox_4 gene in groups F, G, H, and C ($P < 0.05$) (Figure 1).

Table 4 demonstrates the Mean ± SEM of the expression of the Nox_4 gene in mice treated with magnesium sulfate, 24 h after irradiation. There was a significant difference between the expression of the Nox_4 gene in group C and group E ($p < 0.05$); there were also significant differences in the expression of the Nox_4 groups I, J, K, and group C ($P < 0.05$) (Figure 2).

Table 3. Mean ± Standard Error of Mean (SEM) of Nox_4 gene expression in different groups

Group code	Fold change
B	0.35 ± 0.0011
C	15.94 ± 0.011
D	0.38 ± 0.017
F	1.71 ± 0.011
G	0.99 ± 0.00088
H	1.66 ± 0.00058

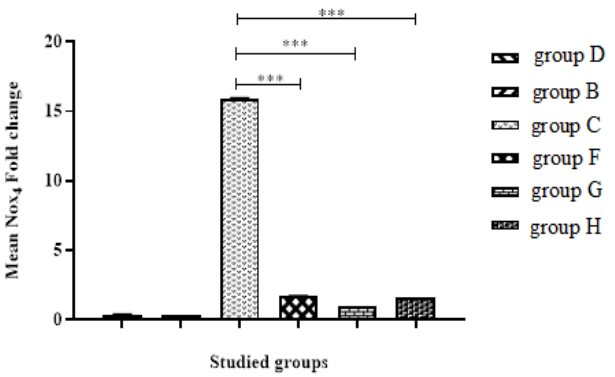

Figure 1. Mean expression of Nox_4 gene (fold change) in various groups, 24 h after irradiation

Table 4. Mean ± Standard Error of Mean (SEM) of Nox_4 gene expression in various groups

Group code	Fold change
C	15.94 ± 0.011
E	0.82 ± 0.31
I	2.48 ± 0.0011
J	0.99 ± 0.0011
K	0.77 ± 0.023

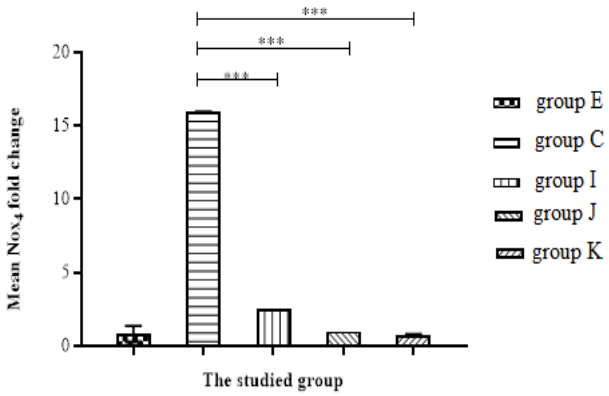

Figure 2. Mean expression of Nox_4 gene (fold change) in different groups, 24 h after irradiation

4. Discussion

The detrimental effects of IR on targets can occur in two direct and indirect mechanisms [29]. In an indirect way, ionizing radiation collides with water molecules of tissues resulting in a reduction of oxygen (O_2), and generation of free radicals such as H, eaq-, OH, and ROS. Although the half-life of free radicals is very short, they react with cellular macromolecules, such as lipids, DNA, carbohydrates, and proteins causing oxidative damage. Hence, damage to DNA by ionizing radiation is chiefly because of oxidative stress [30].

ROS are active chemical species that contain oxygen, such as superoxide, singlet oxygen, hydroxyl radicals, and peroxides [31]. Though ROS generally exists in cells, when water molecules were ionized by radiation, they produce surplus ROS, and increase the amount of ROS within the cell [32]. Thus, if antioxidants are given to the tissues before irradiation, the amounts of intracellular

ROS diminish both in vivo and in vitro, and finally protect cells [33].

The origin of ROS within the cells has not yet been recognized. Three likely sources are disrupted mitochondria, ROSs produced by mitochondria after the DNA being damaged by radiation, NOX/DUOX family. The NOX/DUOX family has 7 members, including $DUOX_1$, $DUOX_2$, and Nox1 to Nox_5. NADPH oxidase conveys an electron from NADPH to molecular oxygen, thus generating a superoxide anion that is a precursor to other free radicals of oxygen and nitrogen [34, 35].

In our study, the highest expression of the Nox_4 gene was in group C, which represents irradiation enhances the expression of the Nox_4 gene. It has been shown that the expression of the Nox gene increases in the endothelial cells of the brain in rat following irradiation [36]. Pazhanisamy *et al*. (2011) also proved that genomic instability of the hematopoietic system decreases when the expression of the Nox gene had been blocked by diphenylene iodine after whole-body irradiation of 6.5 Gy [37]. In addition, it has been shown that chronic oxidative stress and Nox activity raised after irradiation. Chatterjee *et al.* indicated that the expression of Nox_4 was upregulated after irradiation and also irradiation caused an increase in the level of ROS [38]. These findings hypothesize that decline in Nox activity can diminish radiation-induced DNA injury and even cell death. Therefore, the finding of our study is in accordance with the results of other investigations.

Some research has demonstrated that radioprotectors can decline the expression of the Nox_4 gene. Najafi *et al.* indicated that whole-body irradiation upregulates the expression of the Nox_4 gene and melatonin downregulated the expression of the Nox_4 gene in non-target and target cells [39]. In addition, Jiang *et al*. showed that naringenin diminished the expression of the Nox_4 gene and protected the healthy tissues against radiation [40]. Furthermore, Yang *et al.* found that the expression of the Nox_4 gene was boosted after irradiation, and treatment with magnesium isoglycyrrhizinate decreased the expression of the Nox_4 gene in normal tissues [41]. Therefore, it can be understood that the reduction in the Nox_4 gene expression can be used as a base to determine the radioprotective rate of substances.

The results of our study indicated that vitamin A and magnesium sulfate can decrease the expression of the Nox_4 and play a radioprotective role against irradiation. In all three concentrations of $mgso_4$ + radiation, the differences between only the radiation group and treatment groups were significant. Besides, the most effective concentration of vitamin A and magnesium sulfate are 200 mg/kg and 300 mg/kg, respectively.

Generally, the radioprotective effect of vitamin A is related to its antioxidant characteristics, which barricade the pathways of reactions stimulated by free radicals [42]. Retinol, also called Vitamin A1, neutralizes free radicals by preventing peroxidation in a homogeneous methyl linoleate solution. Vitamin A is oxidized by free radicals of peroxyl, generating 5,6-epoxy retinoic and finally nullifying free radicals [43]. In addition, the mixture of vitamin A and soybean oil-soluble vitamin A has been proved to have a radioprotective function against radiation injury caused by internal radionuclides [44].

In our study, the differences between groups treated with magnesium sulfate and only the radiation group were significant, which is in line with the results of previous investigations. It has been indicated that the rate of oxidative stress increases when there is an insufficiency in magnesium quantity of cells, and treatment with magnesium can increase the integrity of DNA, protein production, enzymes involved in protein biosynthesis, and gene transcription [45, 46]. Furthermore, it has been indicated that magnesium is able to offset free radicals because of its antioxidant role [47-49]. Geiger *et al.* (2012) found that deficiency in magnesium augments insulin resistance, glucose resistance, and blood pressure by decreasing oxidative stress [50].

5. Conclusion

All in all, it can be implicated that magnesium sulfate can scavenge free radicals because it is an antagonist of calcium and also has antioxidant properties. However, more research should be done in order to clarify its radioprotective efficiency. In conclusion, the results of this research indicated that magnesium sulfate and vitamin A can diminish the expression of the Nox_4 gene following 2 Gy X-ray irradiation. This study also proposes that vitamin A and magnesium sulfate might be able to protect the bone marrow tissues of mice against injuries caused by ionizing radiation.

5.1. Limitations/Suggestions

There were some limitations such as applying different doses of radiation on tumors, then measuring the

radioprotective effects of the drugs on healthy and cancerous tissues. Therefore, it is highly recommended to evaluate the radioprotective effects of vitamin A and magnesium sulfate in tumor cells in the next studies.

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