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The Radioprotective Effect of Magnesium Sulfate and Vitamin A on Radiation-induced Micronuclei and the Expression of NOX4 in Bone Marrow Cells of Mice

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ABSTRACT

Background: Radioprotectors are used to neutralize the effects of free radicals caused by ionization radiation. In this study, the radioprotective effects of magnesium sulfate and vitamin A on bone marrow cells of mice were evaluated by micronucleus assay and changes in the expression of NOX4 gene.

Methods: The mice were randomly divided into 12 groups. The mixture of drugs was injected into mice by intraperitoneal injection 2 hours before the irradiation. The dose rate was 50 cGy/min at SSD (source to surface distance) 100 cm and field size of 10×10cm^2. Twenty four hours after 2 Gy irradiation by LINAC, the mice were sacrificed by cervical dislocation. Then, several microscopic slides were prepared for each sample to evaluate the number of micronucleus in polychromatic erythrocytes (PCEs). In addition, the expression of NOX4 was evaluated by Real-time PCR. Data were analyzed through SPSS 19 and the mean of groups was compared to each other using one-way ANOVA.

Results: There was a significant difference between mean mnPCEs in the treatment (drugs + radiation) groups compared to the 2 Gy group (P=0.01). The expression level of NOX4 gene was significantly lower in groups receiving the combinations of vitamin A and magnesium sulfate compared to the 2 Gy group (P =0.01). The calculated dose reduction factor (DRF) demonstrated DRF=2.58 for 2Gy.

Conclusion: The results of this study indicated that the combination of vitamin A and magnesium sulfate, possibly with an antioxidant mechanism, removes the deleterious effects of free radicals caused by ionizing radiation on bone marrow cells.

Keywords: Micronucleus assay, Magnesium sulfate, NOX4, Radiation-protective agents, Vitamin A

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Introduction

Ionizing radiation (IR) is one of the most threatening natural, occupational and medical agents that can bring about serious health damages (1). Although the dose of the occupational radiation is generally low, it could increase exceedingly due to the radiological accidents at nuclear reactors (2-4), exposure to radioactive waste (5), outcomes of nuclear bombing (6), the industrial accidents in the course of mining and processing of radioactive substances (7). In these cases, the risk of exposure to the great amount of radiation can reach a significant extent not only for radiation employees but also for personnel engaging in emergency response.

Clinical application of IR is broadly used for the treatment of a wide variety of cancers as a part of therapy. Half of all cancer patients are expected to undergo radiotherapy at some point in in the process of their cancer treatment. Previous studies have shown that augmenting the cumulative radiation dose by 10-20% can completely eradicate some tumors (8). However, high cumulative radiation dose can harm healthy tissues surrounding the tumor and therefore brings about side effects.

In order to protect the normal tissues from detrimental effects of IR, many radioprotectors and mitigators have been developed (9-12). Mitigators are used to decrease toxicity and applied even after irradiation (13,14). Radioprotectors are substances that are able to diminish the harmful effects of radiation on normal tissues and they should be present in the tissues before or at the time of irradiation (15).

Radioprotective agents that have been so far suggested are categorized into three groups; thiol compounds that are capable to neutralize the generated free radicals by radiation, cytokines and growth factors that alter cellular reaction to radiation by moderating communication between immune cells and other kinds of cells, and herbal extracts and natural antioxidant (16). The most common and effective radioprotectors are thiol substances. Amifostine, a thiol compound, is the only radioprotector that has been approved by the US food and drug administration (FDA). Nonetheless, it has adverse effects including vomiting, nausea and hypotension. Due to the toxicity of amifostine, its applications in clinical trials is considerably low compared to that in animal investigations (17). Another group of radioprotectors that have recently been considered is natural and herbal antioxidants, such as melatonin, vitamin C, flavonoids and so on. These natural compounds have fewer radioprotective effects and lower adverse effects compared to the thiol compounds. One approach to increase the effectiveness of radioprotectors and diminish their toxicity is to investigate natural radioprotectors, such as vitamins and magnesium sulfate (18). Vitamin A is one of the fat-soluble vitamins and is essential for the growth of the body, the proper functioning of the immune system, and the prevention of infections (19). Vitamin A does not exist purely in plant sources, but in its precursors, carotenes, in various forms. The most common precursor of vitamin A is beta-carotene, some of which is converted to vitamin A in the body. Beta-carotene effectively reduces the production of trichloromethyl peroxyl radicals and protects the membrane against lipid peroxidation (20). Previous studies have found that magnesium has antioxidant effects, and its ability to reduce and scavenge free radicals has been of interest to researchers (21-23). Magnesium inhibits nicotinamide adenine dinucleotide phosphate oxidase (NOX), which increases the production of oxidized free radicals. As a result, magnesium can directly inhibit the production of free radicals or scavenge free radicals (24, 25).

There are several assays to assess the efficacy of radioprotectors, such as Micronucleus (MN) assay, dicentric assay and gene expression method (26). MN is formed by the break of chromosomes at the anaphase stage of mitosis in immature polychromatic erythrocytes (PCE). The development of immature erythrocytes or PCE into mature erythrocytes or normochromatic erythrocytes (NCE) takes about 6-7 cell divisions. Using the MN method, chromosomal damages that change the number and shape of chromosomes can be counted (27).

Although the dicentric assay is currently the gold standard for biodosimetry, it is not possible to use this technique at high mass casualty rates without automatic dicentric methods. Even with automated counts, dicentric counts require cell division, so it takes at least 3 days for the results to be ready (28). Since gene expression does not require cell division, it can estimate the dose within hours (29). Exposure of human cells to environmental stress including IR activates several cellular signal pathways and rapidly leads to complex patterns of gene expression. Expression of specific genes depends on radiation dose and the kind of oxidative stress.

Gene expression changes may continue for several days after radiation exposure. These gene expression changes can be used to estimate radiation dose and radiation damages (30). In addition, changes in the expression of specific genes including NOX4 in the control and case groups can approximately estimate the radioprotective property of drugs.

NOX gene encodes a member of the NOX enzyme family that acts as an oxidase. NADPH is a catalytic subset of the oxidase complex of nicotinamide adenine dinucleotide phosphate oxidase, an NADPH membrane enzyme complex that faces the extracellular space. This encoded protein binds to non-phagocytic cells, where it acts as an oxygen sensor and catalyzes molecular oxygen to reactive oxygen species (ROS). The ROS produced by this protein has been implicated in various biological functions including signal transduction, cell differentiation and tumor cell growth (31).

The role of NOX4 in many cancers, such as glioma, melanoma, and thyroid cancer is also significant. It has been shown that NOX4 contributes to the progression of metastasis in various cancers (32). Other studies have shown that NOX4 regulates the cell cycle, decreases proliferation, and increases cell apoptosis (33, 34). Measuring changes in the expression of this gene in the groups of animals given radioprotective drugs compared to the control group, along with the MN method can give us more information to more accurately estimate the radioprotective properties of the substances.

Given the adverse effects of radiation in radiotherapy and the lack of appropriate radioprotectors, such as amifostine due to its side effects, further research is needed to achieve radioprotective compounds with fewer side effects and higher radioprotective properties. Therefore, the aim of this study was to investigate the radioprotective effect of magnesium sulfate and vitamin A in combined doses to find out whether magnesium sulfate and vitamin A have synergistic effects on the radioprotective activity of each other.

Materials and Methods

Grouping of animals

In this study, 6-7 weeks old NMRI male mice were used. They were kept at a suitable temperature and 12/12 light cycle. The mice were categorized into 12 groups so that each group contained 5 mice (Table 1). In addition, the ethical code from the Ethical Committee of Tehran University of Medical Sciences was obtained, animals were also treated in accordance with Guide for the Care and Use of Laboratory Animals (8th edition, National Academies Press) (35). The Ethical Committee for medical Research at Tehran University of Medical Science, endorsed this research [ethical code IR.TUMS.SPH.REC.1396.4098].

Prescription of drugs

The radioprotective drugs were Vitamin A (DarouPakhsh Pharmaceutical Co, Tehran, Iran) and magnesium sulfate (Merck, Germany, pa). The mice were given 9 different combined doses of vitamin A (100, 200 and 400 mg / kg) and magnesium sulfate (75, 150 and 300 mg/kg) to determine the optimal dose. Vitamin A was dissolved individually in ethanol (%5). Then different combined doses of drugs were injected intraperitoneally into the mice by insulin syringes. Also, ethanol (%5) that is the solvent of vitamin A was injected into a group of mice to realize the difference between control group (group A) and ethanol group (group B). Mice were placed in standard irradiation cages and exposed to 2 Gy (whole-body irradiation) of X radiation by 10 MV x-ray beams from a linear accelerator (*Varian 2100 CD*). The dose rate was 50 cGy/min at SSD (source to surface distance) = 100 cm and *field size of* $10\times10cm^2$ (36).

Bone marrow sampling

Twenty four hours after irradiation, the animals were anesthetized and sacrificed by cervical dislocation. Then, both femoral bones were removed, the bone marrow of each femur was extracted by 1 cc fetal bovine serum (FCS) from the lower end of the femur and transferred into the microtube. For each mouse, the bone marrows of both femurs were extracted into two microtubes. One microtubule for MN test and the other for evaluation of gene expression.

MN test

The bone marrow cells were centrifuged at 2000 rpm for 6 min at 4 ° C; then a series of the bone marrow cells was placed at -70 ° C for RNA extraction and the other for the MN technique. The cells were afterward transferred to the microscopic slides and fixed for 5 minutes using the methanol solution, dried by exposure to open air for 24 hours and stained with May-Grünwald-Giemsa staining solution. To determine the number of MN in each sample, 1,000 PCEs were

counted by a Y100 Nikon microscope with 100× objective lens (37,38).

Gene expression method

Generally, gene expression consists of three stages; extraction of RNA from bone marrow cells, cDNA synthesis (RT-PCR) and Real-time PCR. To evaluate the expression of NOX genes in tumor tissues, RNA was extracted using RNeasy Mini Kit (Qiagen). Also, its quality and quantity were assessed using agarose gel (1.5%) and spectrophotometer (Thermo Scientific™ NanoDrop-1000), respectively. To perform cDNA synthesis (RT-PCR), 2 micrograms RNA were converted into cDNA by SuperScript II reverse transcriptase (Invitrogen); in addition, Oligo $(dT)_{15}$ primer (Roche) that adhere to the poly A of mRNAs was used. To carry out Real-time PCR, several materials including characteristic primers of Nox4 [Table 2], *Light Cycler® FastStart DNA Master^{PLUS} SYBR Green I, a* Light Cycler Real-time machine (Roche) were used. GAPDH gene was used as an internal standard (Table 2). To compare the relative quantities (RQ) of gene expression between treatment groups and control group, the fold change of the gene was calculated using the comparative CT method, known as the $2^{-\Delta\Delta Ct}$ method (39).

Statistical analysis

Normal distribution of data was performed using histogram in SPSS 16; the mean MN and gene expression were expressed as Mean ± SE. Then, to compare means of MNPCEs and means of gene expression changes in groups, one way ANOVA was used. Also, the differences between the means of different groups were determined by Tukey's Post Hoc Test ($P < 0.05$).

Results

Statistical analysis showed that the combination of vitamin A and magnesium sulfate had a significant radioprotective effect. The MnPCEs/1000PCEs was 98.66 ± 3.05 in 2 Gy x-ray group; however, it was 34 ± 2 in group H (table 3). There was a significant difference (P=0.01) between mean mnPCEs in the treatment (drugs + radiation) groups compared to the 2 Gy group C (table 3). The calculated dose reduction factor (DRF) demonstrated DRF=2.58 for 2Gy (table 3).

Mean ± standard error of mean (SEM) of NOX4 gene expression was 15.94±0.011 in 2 Gy x-ray group; although, it decreased to 0.76±0.052 in group H (table 4). The expression level of NOX4 gene was significantly lower in groups receiving the combinations of vitamin A and magnesium sulfate compared to the 2 Gy group C (P =0.01). But, the differences in the mean expression of Nox4 in the group D, group E, and group H compared to the group C were greater (P= 0.001). The highest difference in Nox4 gene expression was observed between 2Gy+200 mg/kg vit A+150 mg/kg $mgso_4$ group and 2 Gy Group C (table 4).

Table 1. The division of animals into the 12 studied groups

Group code	Dose of X radiation (Gy)	Dose of drugs (mg/kg)
A	0	0
B	0	5 cc ethanol (5%)
C	2	0
D	2	100 vit A + 75 $mgso_4$
E	2	100 vit A + 150 $mgso_4$
F	2	100 vit A + 300 $mgso_4$
G	2	200 vit A + 75 $mgso_4$
H	2	200 vit A + 150 $mgso_4$
I	2	200 vit A + 300 $mgso_4$
J	2	400 vit A + 75 $mgso_4$
K	2	400 vit A + 150 $mgso_4$
L	2	400 vit A + 300 $mgso_4$

Table 2. Forward and reverse primers of Nox4 and GAPDH genes

Primer	Sequence
GAPDH F	5-CCCTTAAGAGGGATGCTGCC-3
GAPDH R	5-TACGGCCAAATCCGTTCACA-3
NOX4 F	5- TTGCCTGGAAGAACCCAAGT -3
NOX4 R	5- TCCGCACAATAAAGGCACAA -3

Table 3. Mean±SE frequencies of MnPCEs/1000PCEs in bone marrow in various groups, 24 hours after 2 Gy of X radiation

Groups	MnPCEs/1000PCEs	Dose Reduction Factor (DRF)
A	20 ± 2.16	
B	31.80 ±3 .27	
C	98.66 ± 3.05	
D	53.66 ± 4	1.83
E	33 ± 2	2.98
F	33 ± 2	2.98
G	37.33 ± 3	2.64
H	34 ± 2	2.90
I	34 ± 4	2.90
J	45 ± 4	2.19
K	49.33 ± 8	2
L	35 ± 1	2.81

Table 4. Mean ± standard error of mean (SEM) of NOX4 gene expression in different groups

Groups	Fold change
C	15.94±0.011
D	1.53±0.011
E	3.02±0.25
F	1.59±0.20
G	4.27±0.018
H	0.76±0.052
I	2.85±0.023
J	2.85±0.023
K	3.52±0.33
L	8.81±1.78

Discussion

In the present study, the expression of NOX4 gene was highest in the group receiving only 2 Gy X-ray, indicating that irradiation increases the expression of NOX4 gene. In Collins-Underwood *et al.* study, the expression of NOX gene increased in the brain endothelial cells of rats after 10 Gy irradiation (40). Moreover, Pazhanisamy *et al.* also found that inhibition of Nox gene by the diphenylene iodine after whole-body irradiation of 6.5 Gy reduces the genomic instability of the hematopoietic system (41). Irradiation has been shown to induce chronic oxidative stress response to NOX activity in rat cells. This finding suggests that reduction in the expression of NOX could be a sign of decline in radiation-induced DNA damage and cell death (42). Therefore, the result of our research is in line with the results of previous studies.

Some investigations have indicated that radioprotectors can decrease the expression of Nox4 gene. In Najafi *et al.* study, whole-body irradiation increased the expression of NOX4 gene and melatonin reduced the expression of NOX4 gene in target and non-target lung tissues (43). Moreover, Yang *et al.* figured out that the expression of Nox4 gene increased in radiotherapy of fibrosis, and treatment with magnesium isoglycyrrhizinate diminished the expression of Nox4 gene and protected healthy tissue against radiation (44). In addition, Jiang *et al.* found that X-ray irradiation of mice enhanced the expression of NOX4 gene and naringenin treatment reduced the expression of this gene and protected the animal's healthy tissues against radiation (45). Based on the results of the mentioned studies, it can be concluded that the reduction of NOX4 gene expression can be considered as a criterion to compare the protective effect of radioprotective agents.

The results of our study showed that injection of vitamin A and magnesium sulfate in the mixed doses can have a radioprotective effect against 2 Gy X-ray. In both methods, gene expression and MN assay, the differences between treatment groups and the 2 Gy X-ray group were significant. Based on the results of gene expression technique, the most effective combination of vitamin A and magnesium sulfate was 200 mg/kg and 150 mg/kg, respectively. Also, based on the results of MN technique, the most effective dose has a dose reduction factor (DRF) of 2.9, which is consistent with the results of the gene expression technique.

In general, the radioprotective mechanism of vitamin A is attributed to its antioxidant properties. Vitamin A plays a protective role against radiation by blocking the pathways of chain reactions initiated by free radicals (46). The most common precursor of vitamin A is beta-carotene, which effectively reduces the produced trichloromethyl peroxyl free radicals and protects the membrane against lipid peroxidation (47). Retinol scavenges free radicals by inhibiting peroxidation in a homogeneous methyl linoleate solution. To stabilize and neutralize peroxyl free radicals, vitamin A is oxidized by these free radicals, producing 5, 6-epoxy retinoic and ultimately stabilizing free radicals (48). Soybean oil-soluble vitamin A has been shown to protect healthy tissues from the radiation damage caused by internal radionuclides (49).

The radioprotective effect of magnesium sulfate in both MN assay and gene expression method was significant. Studies have shown that the insufficiency of magnesium increases the

rate of oxidative cell death, and magnesium can augment the stability of DNA, the maintenance of enzymes involved in protein biosynthesis, gene transcription, protein production, and cell growth. Magnesium also plays a fundamental role in the structure and physiology of cells (50, 51). Magnesium has been shown to have a strong anti-inflammatory capacity as well as an antioxidant role against free radicals (52). Geiger *et al.* showed that magnesium plays a role in preventing oxidative stress so that insufficient magnesium increases blood pressure, glucose resistance, and insulin resistance (53). Since magnesium is a natural antagonist of calcium and also has antioxidant effects, it is likely that magnesium sulfate scavenges free radicals using antioxidant properties. Nonetheless, further research is needed to determine the extent of its radioprotective effect.

The greatest difference of means was observed between group H (2GY radiation + 200mg/kg Vit A + 150mg/kg $mgso_4$ and group C (2GY radiation+ no treatment). The results of this study show that the radioprotective effect of the combination of two drugs, vitamin A and magnesium sulfate, is promising. The optimal combination dose of the two drugs in whole-body irradiation is found in group H.

There were some limitations in our work such as using only two radiobiological dosimetry methods to evaluate the radioprotective effects of the drugs due to low budget of the project. We recommend other researchers to carry out more experiments on the radioprotective effects of vitamin A and $mgso_4$ on tumoral and normal tissues using different doses of x-ray.

Conclusion

In summary, this study showed that the radioprotective effect of the combination of two drugs, vitamin A and magnesium sulfate, were relatively high for protection against 2 Gy X-ray. In addition, the expression of NOX4 gene and the number of mnPCEs in bone marrow cells increased by 2 Gy X-ray irradiation. The results of this study also suggest that the combination of magnesium and vitamin A on the expression of NOX4 gene may protect the bone marrow cells of mice against IR damage. Thus, a mixture of both vitamin A and magnesium sulfate can be used as a radioprotective agent in patients undergoing radiotherapy, occupational exposure, nuclear accidents and space travel.

Conflict of interest

Not declared.

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