




Radioprotective Potential of Sulindac Sulfide to Prevent DNA Damage Due to Ionizing Radiation

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Introduction: The ionizing radiation exposure of the normal cell causes damage to DNA, which leads to cell dysfunction or even cell death. However, it is necessary to identify new radio protectives in order to protect normal cells. Sulindac sulfide (SS) is a metabolite of sulindac (a non-steroidal anti-inflammatory drug) known as a cyclooxygenase inhibitor. Free radicals and reactive oxygen species are generated in the IR-exposed cells. Also, the induced inflammation process causes damage in DNA.

Purpose: In this research, the radioprotective effect of SS was investigated against genotoxicity and lipid peroxidation induced by ionizing radiation in the human blood lymphocytes.

Methods: In this study, the human blood samples were pretreated with SS at different concentrations (10, 25, 50, 100 and 250 μ M) and then were exposed to IR at a dose of 1.5 Gy. The micronucleus (MN) assay was used to indicate the radioprotective effects of SS on exposed cells. Total antioxidant activity of the SS was measured by using FRAP and DPPH assay. Also, the malondialdehyde (MDA) levels and the activity of superoxide dismutase (SOD) on the exposed cells were evaluated.

Results: It was found that SS decreased the percentage of MN induced by IR in exposed cells. Maximum reduction in the frequency of MN was observed at 250 μ M of SS (87%) that provides the highest degree of protection against IR. On the other hand, pretreatment at 250 μ M of SS inhibited IR-induced oxidative stress, which led to a decrease in the MN frequencies and MDA levels, while SOD activity showed an increase in the exposed cells.

Conclusion: It could be concluded that SS as a good radioprotective agent protects the human normal cells against the oxidative stress and genetic damage induced by IR.

Keywords: sulindac sulfide, DNA damage, MN, radioprotective, genotoxicity, lipid peroxidation

Introduction

Radiotherapy is commonly used in the treatment of a wide variety of malignancies. Radiation is the most important non-surgical method to cure the tumor. Nearly half of all cancer patients are given radiation during the course of their disease. Ionizing radiation, IR of normal tissues, may result in both acute and chronic toxicities that can further cause a range of symptoms and a decrease in quality of life. Radioprotectors are known as antioxidants that decrease the damage to the normal tissues by radiation.¹

IR can increase DNA damage and produce stress response and inflammation; it can even directly or indirectly cause cell death and carcinogenesis. As a direct effect, DNA molecules are hit directly by the radiation^{2,3} that leads to SSB (single-stranded-binding proteins) and DSB (double-stranded-binding proteins) formation.⁴ In indirect effect, the

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ionization of the water molecules produces reactive oxygen species (ROS) such as $\text{OH}\cdot$, H_2O_2 , OH^- , O_2^- that increase free radicals and other reactive species.⁵ Increase in the lipid peroxidation indicates the ROS-dependent cellular damage.⁶ ROS cause the release of arachidonic acid from membrane phospholipids and may increase the formation of prostaglandins and leukotrienes.⁷ Therefore, ROS are known as mediators of inflammation in vivo. Also, there is a direct relation between ROS-mediated inflammation and DNA damage.⁸

As previously mentioned, ionizing radiation with several mechanisms can cause damage to cells including the production of free radicals, the reduction of antioxidant stores inside the cell and the development of inflammatory processes inside the cell. Ionizing radiation increases inflammatory markers such as cytokines and interleukins, and the increased inflammatory processes activate some of the intracellular pathways and signals, which ultimately induce or exacerbate radiation damage to the cell's DNA. There are complex and reasoned connections between the production of free radicals, the increase in inflammatory processes and DNA damage in the exposed cells, in such a way that each of these free radicals and inflammatory processes reinforces the other and increases the damage.^{9,10} Therefore, radioprotectors are often drugs or compounds with free radical scavenging (antioxidant) or anti-inflammatory activity that showed the role of radiation protection inside the cell.^{11–14}

Sulindac is an inactive non-steroidal anti-inflammatory drug (NSAID) that is quickly metabolized following oral administration (Figure 1). Sulindac has two metabolites, sulindac sulphone as an inactive metabolite that does not inhibit COX, and sulindac sulfide as the pharmacologically

active metabolite that has NSAID properties and inhibits COX. Sulindac sulfide can directly inhibit 5-lipoxygenase and can also penetrate into the lipid bilayer. Furthermore, it can ionize the carboxyl group of the membrane, which shows better antioxidant capacity at the membrane level and potentially protects normal cells from ROS implicated in the pathophysiology of inflammation.^{15,16} Thus, sulindac sulfide protects the membrane against oxy-radicals and reduces the expression of pro-inflammatory factors $\text{TNF}\alpha$, iNOS, IL-1 β and IL-6.^{17,18}

It has been reported that anti-inflammatory agents are able to mitigate pro-inflammatory biomarkers involved in IR-induced cellular toxicity.^{19–21} NSAIDs could protect normal tissue by arresting the cell cycle in the G1 state.^{22,23} Furthermore, NSAIDs not only provide radioprotection to normal tissues, but they also offer additive antitumor effects.^{18,24} Therefore, NSAIDs are antioxidants with the potential to minimize cellular damage that their mechanism of action is still unknown.

NSAIDs could elevate the level of superoxide dismutase as an antioxidant enzyme in cells.^{23,25} Therefore, these compounds are able to trap free radicals to avoid DNA damage.²⁶ In the present study, the radioprotective effects of the SS were evaluated on the exposed human lymphocytes by IR.

Materials and Methods

Sulindac sulfide and Cytochalasin-B were purchased from Sigma Chemicals Co. (St. Louis, USA). Phytohemagglutinin (PHA), Roswell Park Memorial Institute (RPMI-1640) medium, fetal bovine serum (FBS), penicillin and streptomycin were purchased from Biosera (USA). Moreover, malondialdehyde (MDA) assay kit (zellbio, Germany) and Superoxide

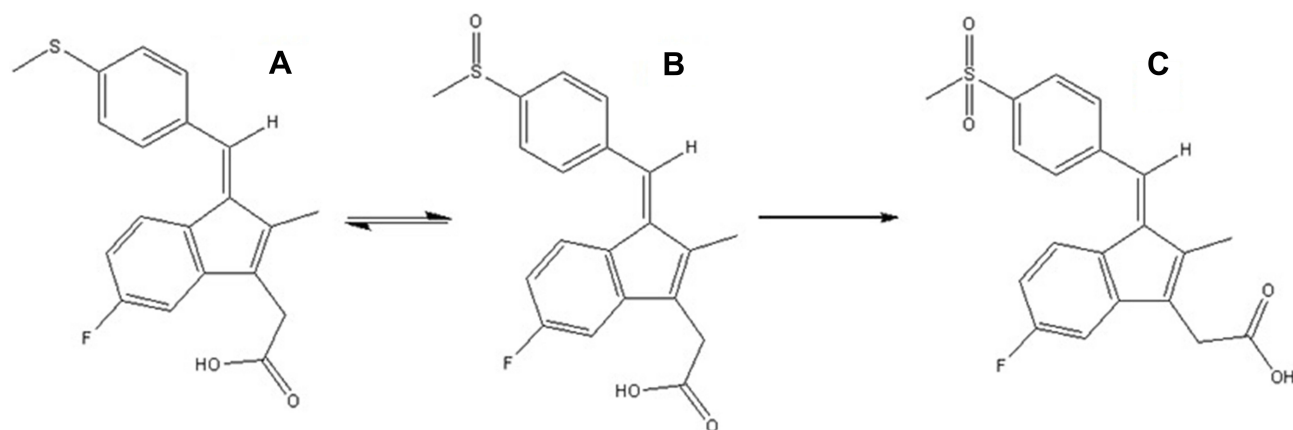


Figure 1 Chemical structure: (A) sulindac sulfide (B), sulindac and (C) sulindac sulfone.

Dismutase (SOD) assay kit (Randox, UK) were purchased. Giemsa stain, methanol and acetic acid were obtained from Merck (Germany).

Determination of Antioxidant Activity of Sulindac Sulfide Using DPPH (1,1-Diphenyl-2-Picrihydrazyl)

The antioxidant activity of the SS was determined using a stable free radical α,α -diphenyl- β -picrylhydrazyl (DPPH). Various concentrations of SS (10, 25, 50, 100, 250 μ M) were mixed thoroughly with an equal volume of ethanol solution of 0.1 M, DPPH. The mixture was allowed to stand for 15 mins in the dark. The absorbance was measured at 517 nm. The experiment was performed in triplicate. Ascorbic acid was used as an antioxidant standard. The scavenging activity was calculated using the following formula:

$$\text{Antioxidant activity}\% = \left[\frac{(\text{AbsControl} - \text{AbsTest})}{\text{AbsControl}} \right] \times 100$$

Total Antioxidant Capacity Assay

The total antioxidant potential of the SS was determined using ferric reducing the antioxidant power (FRAP) assay of Benzie and Strain.²⁷ At low pH, the sample is able to reduce ferric tripyridyltriazine (Fe III-TPTZ) complex to an intense blue-colored ferrous (Fe II) form. This complex has an absorbance maximum at 593 nm and the blue color intensity is proportional to the antioxidant capacity of the sample as described elsewhere. First, 10–250 μ M (5 μ L) of SS and 70 μ L of FRAP reagent were mixed. Distilled water was used as a blank, the mixture was incubated at 37°C for 5 mins and absorbance was read at 593 nm. The FRAP values were expressed as micromoles per liter (μ M) and a standard curve that showed millimole Fe^{2+} to absorbance was used to read these values.²⁸

Blood Treatment

This study obtained permission from research and ethical committees of the Kerman University of Medical Sciences (IR.KMU.REC.1396.2488) and all volunteers provided written informed consent, in accordance with the Declaration of Helsinki. This study enrolled three healthy and non-smoking male volunteers, aged from 21 to 25 years. Twelve milliliters of whole blood were collected in the heparinized tubes and allocated in microtubes each containing 0.9 mL. Blood samples were incubated with

different concentrations of sulindac sulfide including 10, 25, 50, 100 and 250 μ mol/l. These samples were incubated for 2 hrs at 37°C. Control samples were treated with diluted DMSO in RPMI with the same concentration as Sulindac sulfide. DMSO concentration was the same in control and SS solutions (0.1%).

Ionizing Radiation and Micronucleus Test

At each concentration and for each volunteer, peripheral blood samples were irradiated in micro tubes at 37°C with 6 MV photon beam. The photon beam was produced by a medical linear accelerator (Elekta Compact™ Linear accelerator, Crawley, UK) with a total dose of 1.5 Gy delivered in mid-line of microtubes and at a dose rate of 200 cGy/min. Dose calculation was performed using Treatment Planning System (ISOgray TPS, version 5.2, Dosisoft, Cachan, France). Three microtubes were distributed among three volunteers in the control group (non-irradiated samples). Moreover, microtubes containing blood samples were placed on the plastic box filled with water as a phantom, which were exposed to irradiation. Finally, after irradiation, 0.5 mL of each sample (control and irradiated samples in duplicate) was added to 4.4 mL of RPMI 1640 culture medium which contained a blend of 10% FBS and 100 μ L PHA. All cultures were incubated at 37°C in a humidified atmosphere of 5% CO_2 . After 44 hrs, cytochalasin B (final concentration, 6 μ g/mL) was added to the culture. In the subsequent 72 hrs of incubation, the cells were harvested by centrifugation for 10 mins at 3000 rpm and suspended in cold potassium chloride. Then, cells were immediately stabilized in a fixative solution made of methanol:acetic acid (6:1) three times. The fixed cells were spotted onto clean microscopic slides (the triple slide for each concentration), air-dried and stained with 20% Giemsa solution. The slides were coded and evaluated at 100x magnification in order to determine the frequency of MN in the cytokinesis blocked binucleated cells with a well-preserved cytoplasm.²⁹ The MN frequency was determined as 1000 binucleate cells for each volunteer in the treated group. Totally, 3000 binucleated lymphocytes were counted for three volunteers in each treated group, and finally 36,000 binucleated lymphocytes were counted for 12 treated groups in this examination.

Isolation of Lymphocytes

The peripheral blood used in the experiment was obtained from three healthy, non-smoking young male volunteers. Then, blood samples were collected in heparinized sterile

tubes and lymphocytes were isolated by Ficoll-Hypaque using the protocol reported in the previous experiments. Briefly, blood was diluted with an equal volume of a serum-free RPMI medium, layered carefully over Ficoll-Hypaque solution (without intermixing) and centrifuged at 400 g for 30 mins. Then, the lymphocyte layer was aspirated and diluted with serum-free medium and centrifuged at 300 g for 5 mins. The lymphocyte was washed again with serum-free medium and was re-suspended in the RPMI-1640 media. The number of viable cells was determined by the Trypan Blue dye exclusion test. The viable cells were more than 99%.

Determination of MDA

The lipid peroxidation (MDA) was determined using MDA assay kit according to the protocol of the manufacturer (Zellbio, Germany).³⁰ Briefly, butylated hydroxytoluene BHT was added to 10^6 lymphocytes (from every group) and used directly in the assay. Samples (100 μ L) were mixed with sodium dodecyl sulfate and chromogenic solution containing thiobarbituric acid, alkali and acetic acid. All the microtubes were placed on vigorously boiling water for 60 mins. Then, the tubes were shifted to an ice-bath and centrifuged at $\times 3500$ g for 15 mins. The amount of MDA formed in each sample was assessed by measuring the absorbance of the supernatant at 535 nm with an ELISA reader (BioTek Inc., Winooski, USA). Tetramethoxypropane was used as a standard and MDA content was expressed as nmol/mg protein.³¹

The Determination of SOD Activity

The total SOD activity was determined according to the protocol of Randox kit (UK). Superoxide dismutase (SOD) functioned as a catalyst in the dismutation of the superoxide radical (O_2^-) into hydrogen peroxide (H_2O_2) and elemental oxygen (O_2). Lymphocytes cells were harvested and cell lysates were prepared according to kit specifications. The results were read absorbance at 560 nm. Superoxide dismutase enzyme activity level was calculated using the following formula:

$$\text{SOD activation (inhibition\%)} = \frac{(\text{OD}_{\text{blank}} - \text{OD}_{\text{sample}})}{\text{OD}_{\text{blank}}} \times 100$$

Statistical Analysis

For each concentration of SS, the amount of IR-induced micronuclei was recorded for each volunteer. Statistical analysis was performed using one-way analysis of variance (ANOVA) and post hoc Tukey multiple comparison

tests. P value < 0.05 was considered significant and highly significant (SPSS software 16 for windows, 2007, USA).

Results

Free Radical Scavenging

The results of free radical scavenging at different concentrations of SS are shown in Figure 2. The antioxidant radical scavenging activity of SS results was comparable with that of ascorbic acid. The SS and ascorbic acid showed a dose-dependent manner in the scavenging of DPPH free radicals. The higher the concentration of the SS is, the better the percentage of its antioxidant activity will be. At a maximum scavenging activity was recorded at a concentration of 250 μ M of SS and the crude SS antioxidant activity was 62.26 ± 0.20 compared to 71.16 ± 0.40 of ascorbic acid, as an antioxidant standard.

Total Antioxidant Capacity Assay

The results of total antioxidant capacity of SS using FRAP are shown in Figure 3. The antioxidant capacity of SS is

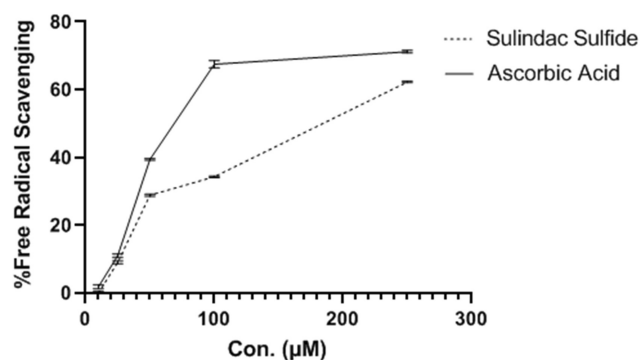


Figure 2 Sulindac sulfide radical scavenging effects at different concentrations at 517 nm.

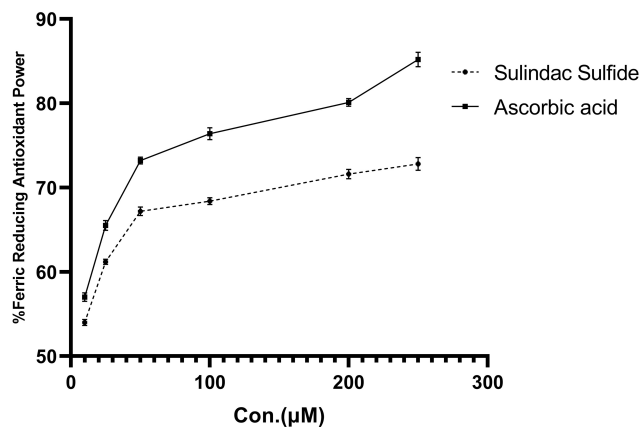


Figure 3 Ferric reducing antioxidant power of the sulindac sulfide.

increased at a gentle slope. It also revealed a concentration-dependent rise up to 250 μM , the highest concentration assessed.

Micronucleus Test

A model for binucleated lymphocyte with micronucleus is shown in Figure 4. The mean percentage of micronuclei in three volunteers treated with 1.5 Gy X-ray was 8.86 ± 0.66 , while it was 0.14 ± 0.05 in non-irradiated control samples.

Exposure of blood samples to IR significantly increased the frequency of micronuclei (40-fold rise) in irradiated lymphocytes ($P < 0.0001$) (Table 1). The frequency of micronuclei after pretreatment with SS at doses of 10, 25, 50, 100, or 250 μM was 7.63 ± 0.76 , 6.9 ± 0.26 , 5.86 ± 0.41 , 1.63 ± 0.11 , and 1.10 ± 0.04 , respectively (Figure 5 and Table 1). The data proved that human blood incubated with SS and then exposed in vitro to X-ray radiation, shows significant reduction in micronuclei frequency compared to blood samples incubated with X-ray alone (without SS, $P < 0.001$). Total micronuclei frequencies in irradiated samples pre-treated with SS were reduced to 13%, 22%, 33%, 81% and 87% at concentrations of 10, 25, 50, 100 or 250 μM , respectively, compared to irradiated samples (Table 1). Amongst irradiated samples with SS, there was no statistically significant difference in micronuclei between doses 100 and 250 μM of SS. The maximum protection of lymphocytes was observed at a concentration of 250 μM through SS treatment. The frequency of MN was significantly reduced in the irradiated sample treated by SS

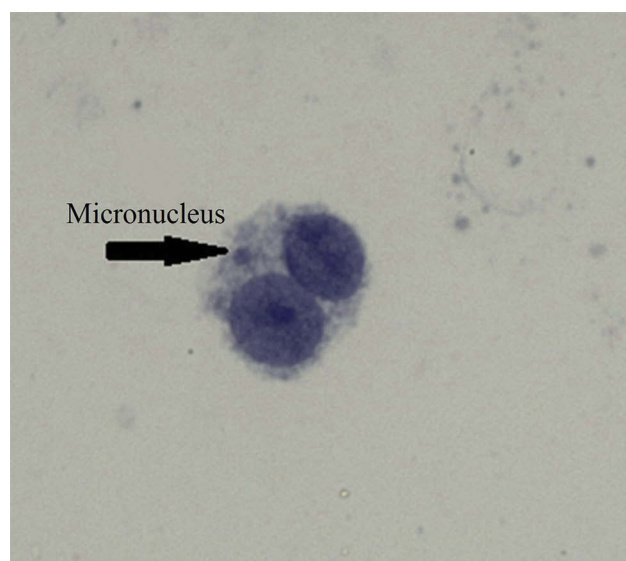


Figure 4 A typical binucleated lymphocyte with micronucleus in our experiment. The arrow shows a micronucleus.

Table 1 The Frequency of Micronuclei Induced in vitro by 1.5 Gy X-Ray Radiation (IR) in Cultured Blood Lymphocytes at Different Doses of Sulindac Sulfide

Volunteers				
Groups	I	II	III	Mean \pm SD
Control	0.2	0.14	0.1	0.14 ± 0.05
IR	8.1	9.3	9.2	$8.86 \pm 0.66^*$
IR+10 SS	6.8	8.3	7.8	7.63 ± 0.76
IR+25 SS	6.6	7	7.1	6.9 ± 0.26
IR+50 SS	5.4	6	6.2	$5.86 \pm 0.41^{**}$
IR+100 SS	1.7	1.7	1.5	$1.63 \pm 0.11^{**}$
IR+250 SS	1.07	1.15	1.1	$1.10 \pm 0.04^{**}$
10 SS	0.3	0.37	0.31	0.32 ± 0.03
25 SS	0.2	0.4	0.35	0.31 ± 0.10
50 SS	0.4	0.32	0.27	0.33 ± 0.06
100 SS	0.2	0.3	0.29	0.26 ± 0.05
250 SS	0.3	0.25	0.18	0.24 ± 0.06

Notes: 10 SS, 10 μM sulindac sulfide; 25 SS, 25 μM sulindac sulfide; 50 SS, 50 μM sulindac sulfide; 100 SS, 100 μM sulindac sulfide; 250 SS, 250 μM sulindac sulfide. 1000 binucleated lymphocyte was examined in each sample, and 3000 binucleated lymphocyte from three volunteers in each group. * $p < 0.0001$ compared to control, ** $p < 0.001$ compared to IR.

Abbreviations: SD, standard deviation; IR, ionizing radiation.

at a concentration of 250 μM as compared to irradiated samples with 10, 25 and 50 μM SS concentration ($P < 0.001$). There was no increased genotoxicity in non-irradiated samples with SS treatments at all concentrations as compared to the control group.

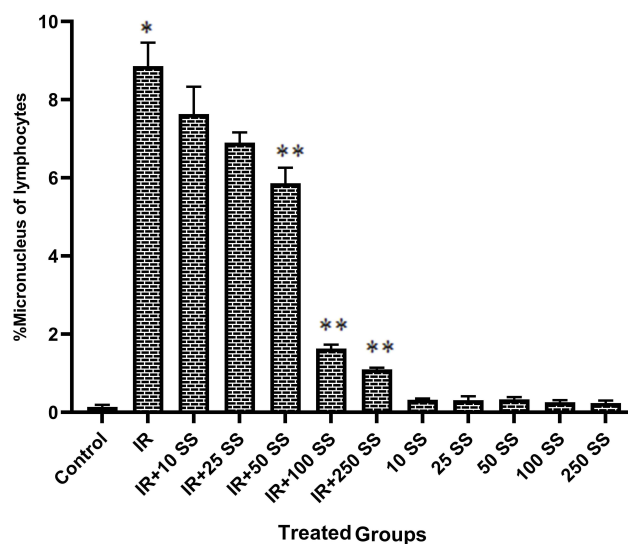


Figure 5 In vitro protection by sulindac sulfide (SS) at different concentrations (10, 25, 50, 100 and 250 μM) against ionizing radiation (IR) induced genetic damage in cultured whole blood lymphocytes. The data represent average \pm standard deviation from three volunteers. * $p < 0.0001$ compared to control, ** $p < 0.001$ compared to IR.

Abbreviations: C, control; 10 SS, 10 μM sulindac sulfide; 25 SS, 25 μM sulindac sulfide; 50 SS, 50 μM sulindac sulfide; 100 SS, 100 μM sulindac sulfide; 250 SS, 250 μM sulindac sulfide.

Lipid Peroxidation

MDA, as an oxidative stress marker, was assayed in the lymphocyte cells. The effect of IR on lipid peroxidation is shown in Figure 6. IR led to a significant increase in the level of MDA compared to control group whereas pretreatment with SS (250 μ M), inhibited LP (lipid peroxidation) in lymphocytes as the concentration-dependent manner. A considerable difference was observed in the MDA level between SS (250 μ M)+IR versus IR alone.

Superoxide Dismutase SOD

Reduced activities of SOD in irradiated lymphocytes were observed in the results of this study (Figure 7). The activity of this antioxidant enzyme was significantly increased after pretreatment with SS (250 μ M) + IR. Hence, the

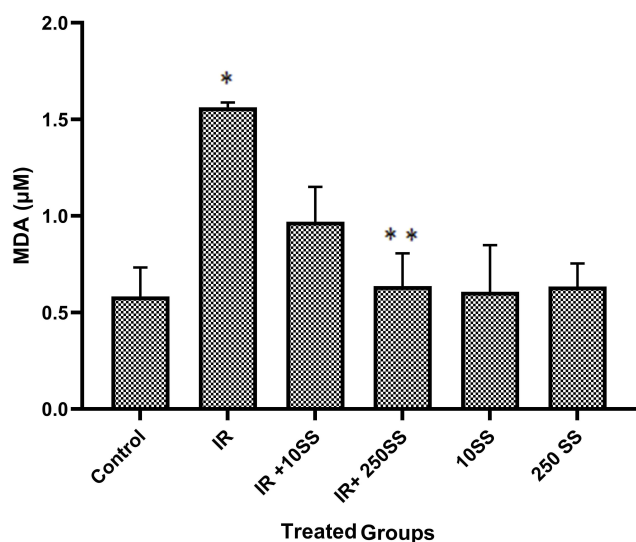


Figure 6 MDA assays on the lymphocytes, * $p < 0.001$ compared to control, ** $p < 0.001$ compared to IR.

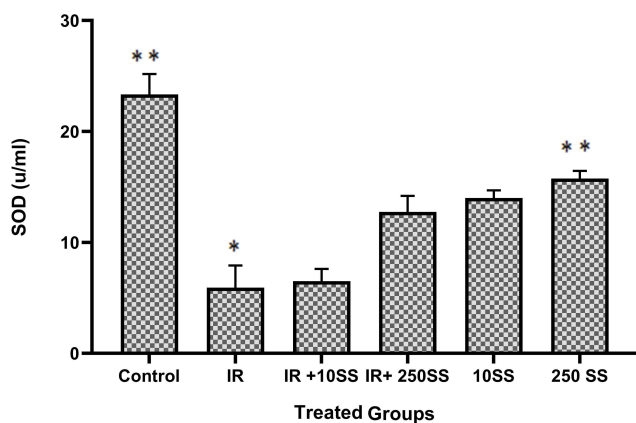


Figure 7 SOD assays on the lymphocytes, * $p < 0.001$ compared to control, ** $p < 0.001$ compared to IR.

results of this study showed that SS increases SOD activity in lymphocytes in a dose-dependent manner.

Discussion

In this study, it was exhibited that priming of human lymphocytes with SS remarkably reduced genotoxicity and stress oxidative induced by IR. The frequency of micronuclei was reduced with pretreatment of SS. Sulindac, as a non-steroidal anti-inflammatory drug (NSAID), is widely used in clinical anti-infection medicine that, in vivo, is reversibly changed to its anti-inflammatory active compound, sulindac sulfide (SS), with the biological effects of inhibition on both cyclooxygenase-1 (COX-1) and COX-2 activities and the decrease in prostaglandin (PG) synthesis.^{32–34} SS was reported as the most active O_2 scavenger which gives reliability to a possible contribution of O_2 scavenging activity for the final therapeutic activity of sulindac.^{35,36} The results of the previous studies demonstrated that SS scavenged HOCl, $O_2\bullet$, $HO\bullet$, $\bullet NO$, $ONOO^-$, and SS are a much more potent $O_2\bullet$ scavenger than sulindac as parent compound. The SS showed that reactive nitrogen species RNS ($\bullet NO$ and $ONOO^-$) and reactive oxygen species ROS ($O_2\bullet$, $HO\bullet$) scavenging activity may contribute strongly to the radioprotective efficacy.³⁷ Therefore, these activity species were decreased by SS.^{37,38} Previous studies investigated that the radical scavengers can be used to protect DNA from free radicals that were generated by radiation.³⁹ In the normal cells, exposure to IR initiates released inflammatory cytokines, which resulted in DNA damage.⁴⁰ Also, NSAIDs could reduce radiation-induced chromosomal instability in vivo.⁴¹ In this study, it was demonstrated that SS relieved IR-induced genotoxicity in human lymphocytes. Clearly, free radicals and inflammation are the main factors for IR-induced DNA damage. A normal cell is going to dysfunction in the inflammatory process. It is documented that the anti-inflammatory effect of SS with inhibition of COX and the decrease in the secretion of cytokines are the main proposed mechanisms for the radioprotective effect of SS. In the present study, IR caused inhibition of SOD activities in irradiated lymphocytes. Since antioxidant enzymes like SOD have the effect of protection against IR, the balance of these enzymes in the cell is important for maximal radioprotection. Here, the SS caused an increase in the activity of SOD in lymphocytes and avoided the accumulation of superoxide radicals and H_2O_2 . ROS and the free radicals influence the membrane lipids and cause extensive membrane lipid peroxidation. Therefore, increased levels of lipid peroxidation induced by IR are accompanied by a decrease in the activity of SOD.³⁸

With its main protective mechanisms including the anti-inflammatory activity, COX inhibition, antioxidant properties, reduction of oxidative stress markers such as LP, and increase of SOD enzyme content, SS can be effective as a radioprotective agent. In fact, there is a crosstalk between rise of oxidative stress level and frequency of MN after the exposure. On the other hand, several researches revealed that the natural agent and anti-inflammatory drugs against oxidant challenge might decrease the rate of mutation and genotoxicity; hence, they helped prevent genotoxic induced by IR.^{19,42,43} This result shows a new indication of SS for the protection of normal cells during radiation therapy in the treatment of cancer patients.

Conclusion

In this study, sulindac sulfide as active metabolite of sulindac with anti-inflammatory and antioxidant properties can decrease genotoxicity and reduce levels of MDA. Moreover, sulindac sulfide can increase the antioxidant activity of SOD enzyme that induced by ionizing irradiation in human lymphocytes. Furthermore, sulindac sulfide showed the low toxicity as non-steroidal anti-inflammatory drugs and free radical scavenging properties. It can help the protection of the body against side effects' ionizing irradiation in human.

Acknowledgments

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Disclosure

The authors report no potential conflicts of interest relevant to this article.

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