Original Article

Effect of heparin-superoxide dismutase on γ-radiation induced DNA damage *in vitro* and *in vivo*

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ABSTRACT: The effects of heparin-superoxide dismutase (SOD) conjugate (heparin-SOD) on γ-radiation induced DNA damage *in vivo* and *in vitro* were evaluated. Plasmid pcDNA3.0 solution was mixed with heparin-SOD, SOD, and a mixture of heparin and SOD (heparin + SOD), respectively, and irradiated with ^{60}Co at a dosage of 120 Gy. DNA injury was analyzed using agarose gel electrophoresis. The results showed that the degree of injury of pcDNA3.0 mixed with heparin-SOD, SOD, or heparin + SOD was less than that of untreated pcDNA3.0, and among them the degree of injury of pcDNA3.0 mixed with heparin-SOD was the least. It also showed that the protective effect increased with an increase of heparin-SOD concentration. The effects of SOD and heparin-SOD on the DNA damage and tumor inhibition rate of ^{60}Co γ-radiation exposure on tumor-bearing mice were also studied. Agarose gel electrophoresis showed that, when different SOD samples were administered before irradiation, the thymus DNA injuries of heparin-SOD, SOD, or heparin + SOD groups were more serious than that of the control group, and the DNA injuries of heparin-SOD or heparin + SOD groups were the most serious, which contradicted the above *in vitro* experiments. However, when heparin-SOD was administered post irradiation, it showed a repairing effect on the injured DNA.

Keywords: Cu,Zn-superoxide dismutase, low molecular weight heparin (LMWH), LMWH-SOD, DNA fragmentation

1. Introduction

Eighty percent of cancer patients need radiotherapy at some time, either for a curative or palliative purpose. Since human tissues contain 80% water, the major radiation damage is due to aqueous free radicals generated by the action of radiation on water. These free radicals react with cellular macromolecules, such as DNA, RNA, proteins, *etc.*, and cause cell dysfunction and mortality. Oxidative damage to the cellular genetic material, *i.e.*, DNA, plays a major role in mutagenesis and carcinogenesis. Highly reactive oxygen radicals produced by ionizing radiation cause lesions in DNA, which lead to cell death and DNA mutation. In order to obtain better tumor control with a higher radiation dose, normal tissues should be protected from radiation injury. Thus, the role of radioprotective compounds is very important in clinical radiotherapy. However, many of them have severe side effects, such as nausea, vomiting, and hypotension (*1,2*).

Enzymes such as superoxide dismutase, glutathione peroxidase and catalase protect mammalian cells from oxidative radiation damage (*3*). Because of the shortcomings of proteins used as medicines, such as short half-life, antigenicity, and instability, the utilization of these enzymes has been limited and increasing attention has been given to chemical modification of proteins to overcome the shortcomings (*4,5*). In our laboratory, Cu,Zn-superoxide dismutase (Cu,Zn-SOD) has been chemically modified with low molecular weight heparin (LMWH), and it was proved that after modification, the immunogenicity was lowered, the anti-inflammatory activity was increased and the stability of SOD towards acid, alkali, heat and trypsin were enhanced (*4*). Our earlier studies showed that heparin-SOD could prevent the effect of carbon tetrachloride-induced acute liver failure and hepatic fibrosis in mice (*6*). Our earlier studies also showed that heparin-SOD could attenuate bleomycin-induced pulmonary fibrosis *in vivo*, and inhibit the inflammatory cytokine expression

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induced by radiation, demonstrating that heparin-SOD might be useful in the treatment of pulmonary fibrosis (*7*). In the present study, the radioprotective effects and possible mechanisms of heparin-SOD *in vivo* and *in vitro* were investigated.

2. Materials and Methods

2.1. *Materials*

Heparin-SOD was prepared according to the method reported previously (*4,5*). Agarose and ethidium bromide were purchased from Sigma-Aldrich, St. Louis, MO, USA. Plasmid pcDNA3.0 was purified from *Escherichia coli* using a Qiagen Plasmid kit-Pack 500 (Qiagen, Hilden, Germany). S180 tumor cells were supplied by Shandong Academy of Medical Science, China. ^{60}Co γ-radiation exposure was performed in the Academy of Agricultural Sciences of Shandong Province, China. Other chemicals and reagents were of analytical grade.

2.2. *Animals*

Pathogen-free male Kunming mice, weighing 23-27 g, obtained from the Experimental Animal Center of Shandong University (Ji'nan, Shandong, China) were used in the experiments. The mice were housed in animal facilities accredited by the Shandong Council on Animal Care and treated in accordance with approved protocols. Animals were maintained in a specific pathogen-free environment that was temperature-controlled (23 ± 2°C) and humidity-controlled (60 ± 10%), under a 12 h light-dark cycle. The animals used in this study were handled and treated in accordance with the strict guiding principles of the National Institutes of Health for Experimental Care and Use of Animals. The experimental design and procedures were approved by the Institutional Ethical Committee for Animal Care and Use of Shandong University, People's Republic of China.

2.3. *In vitro assessment of the radio-protective effect of heparin-SOD on pcDNA3.0*

One microgram of pcDNA3.0 was put into 4 Eppendorf centrifuge tubes, respectively, and 50 μL of SOD, heparin-SOD, a mixture of heparin and SOD (heparin + SOD), and isotonic sodium chloride (as control) were added respectively before irradiation. The added dosages concerning SOD for the above SOD sample were according to Cu,Zn-SOD enzymatic activity (3,000 units/mL), and the heparin dosage in heparin + SOD was the same heparin proportion as in heparin-SOD. After mixing, the four tubes were exposed to γ-radiation at a dose rate of 1 Gy/min on an ice bath with a total dose of 120 Gy as reported elsewhere (*8-10*). The supercoiled (SC) and open circular (OC) forms of DNA were separated using 1.5% agarose gel electrophoresis and DNA bands were quantified by scanning the resulting optical density with a densitometer (LKB Co. Ltd., Stockholm, Sweden) after staining with ethidium bromide (*11*). Radiation induced damage was assessed as an increase in the OC form of DNA (*12*).

In another experiment, the radio-protective effect of heparin-SOD at different enzyme activity levels on ^{60}Co γ-radiation induced pcDNA3.0 damage was assessed. One microgram of pcDNA3.0 was put into 5 Eppendorf centrifuge tubes respectively, and isochoric heparin-SOD solutions containing enzymatic activities of 7.5×10^2, 1.5×10^3, 3.0×10^3, 6×10^3, and 1.2×10^4 units, respectively, were added before irradiation. After mixing, the five tubes were exposed to γ-radiation at a dose rate of 1 Gy/min and a total dose of 120 Gy on an ice bath as above. An equal quantity of DNA (based on optical density measurements at 260 nm) in different tubes was loaded in each lane, and agarose gel electrophoresis was carried out as above.

2.4. *In vivo experiment of the effect of SOD, heparin-SOD, and heparin + SOD on DNA damage and tumor growth inhibition of sarcoma bearing mice caused by ^{60}Co γ-radiation exposure*

To study the effect of heparin, SOD, and heparin-SOD on DNA damage and tumor growth inhibition caused by ^{60}Co γ-radiation exposure *in vivo*, S180 sarcoma bearing mice were prepared by subcutaneous injection of sarcoma cells. The tumor-bearing mice were randomly divided into the following 5 groups (10 mice in each group) on the seventh day after tumor cell transplantation: group I, control (0.5 mL of isotonic sodium chloride); group II, SOD (35,000 units/kg); group III, heparin + SOD (SOD 35,000 units/kg and the same proportion of heparin as in heparin-SOD); group IV, heparin-SOD (35,000 units/kg); group V, no irradiation exposure. Group I ~ IV received an intraperitoneal injection of the corresponding SODs 40 min before radiation exposure. ^{60}Co γ-radiation exposure was at a dose rate of 0.30 Gy/min and the total dose was 6 Gy. Mice were sacrificed by cervical dislocation on the fifth day after radiation exposure. Tumors were removed carefully and weighed. At the same time the thymus was removed for DNA extraction. Agarose gel electrophoresis was carried out to analyze for DNA fragmentation (*11-13*).

2.5. *In vivo experiment of the effect of heparin-SOD injected at different times and different parts of sarcoma bearing mice on the DNA damage caused by ^{60}Co γ-radiation exposure*

S180 sarcoma bearing mice were prepared by subcutaneous injection of the sarcoma cells as above. The tumor-bearing mice were randomly divided into the following 5 groups (10 mice in each group):

group I, control (no radiation administered); group II, received intraperitoneal injection of 0.5 mL of isotonic sodium chloride; group III, intraperitoneal injection of heparin-SOD (35,000 units/kg) 3 min after exposure to γ-radiation; group IV, intraperitoneal injection of heparin-SOD 40 min before exposure to γ-radiation (35,000 units/kg); group V, received intratumoral injection of heparin-SOD 40 min before exposure to γ-radiation. Mice were sacrificed after ^{60}Co γ-radiation exposure as above. The thymus was removed for DNA extraction and agarose gel electrophoresis was carried out to analyze for DNA fragmentation.

2.6. *Statistical analysis*

The differences between the control and experimental groups were analyzed by Student's *t*-test.

3. Results

3.1. *Radio-protective effect of heparin-SOD on pcDNA3.0*

Effects of heparin-SOD, SOD, and heparin + SOD on plasmid pcDNA3.0 damage induced by γ-radiation are shown in Figures 1 and 2. Exposure of plasmid pcDNA3.0 DNA to γ-radiation resulted in broken DNA strands, shown as SC form of plasmid DNA converted to the OC form or linear form. The disappearance of the SC form of DNA could be taken as an index of DNA damage induced by the radiation exposure. The presence of heparin-SOD or heparin + SOD along with pcDNA3.0 during irradiation protected the DNA from radiation-induced lesions as seen in Figures 1 and 2. Employing integral calculus analysis, we found that the DNA damage of the heparin-SOD group was the least. Figures 2A and 2B showed that with the increase of enzyme activity, the SC proportion increased and the OC proportion decreased, which meant that the heparin-SOD protection of DNA damage induced by radiation *in vitro* was concentration-dependent.

3.2. *Effect of SOD, heparin-SOD, and heparin + SOD on thymus DNA fragmentation induced by radiation in vivo*

As shown in Figures 3 and 4, groups treated with intraperitoneal injection of SOD, heparin + SOD, or heparin-SOD 40 min before irradiation showed more serious thymus DNA injury than the control group (Figure 3, lanes 2-4; Figure 4, lane 4). DNA injuries in groups treated with the intraperitoneal injection of heparin-SOD or heparin + SOD were the most serious, which was a contradiction of the above *in vitro* experiment. However, when heparin-SOD was intraperitoneally injected 3 min shortly after irradiation or intratumorally injected 40 min before irradiation, intact thymus DNA was observed (Figure 4, lanes 3 and 6).

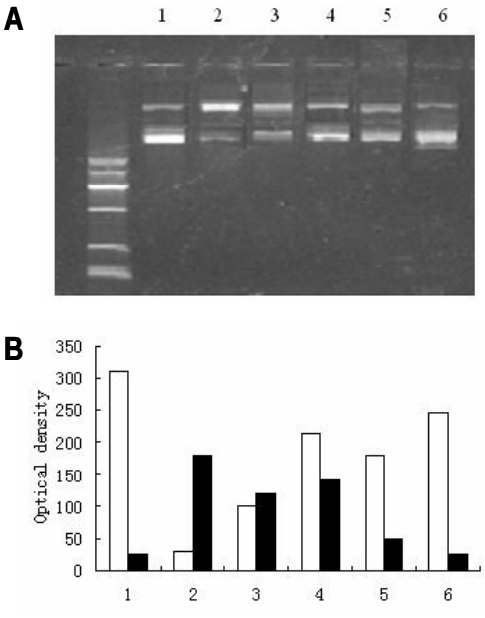

Figure 1. Effect of SOD, heparin-SOD, and heparin + SOD on irradiation induced DNA fragmentation. (A) Agarose gel electrophoresis of pcDNA3.0 after ^{60}Co γ-radiation exposure. The upper and lower bands are the open circular (OC) and the supercoiled (SC) forms respectively. Lane 1, control (no radiation group); Lane 2, isotonic Na chloride group; Lane 3, SOD group; Lane 4, heparin + SOD group; Lanes 5 and 6, heparin-SOD groups. **(B)** Analysis of the pcDNA3.0 agarose gel electrophoresis density integral calculus. Open columns, SC; Closed columns, OC.

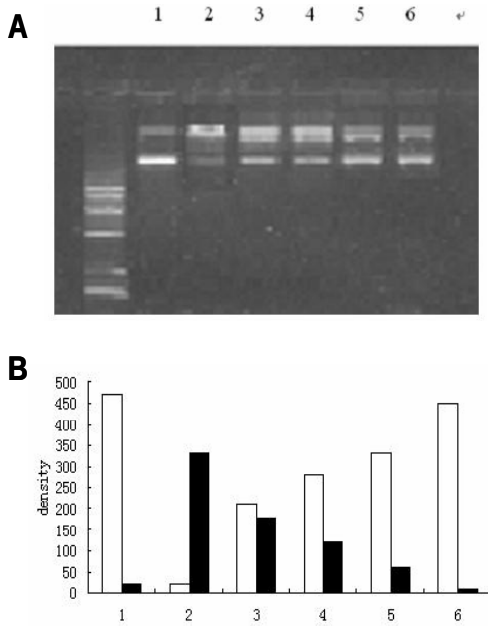

Figure 2. Effect of heparin-SOD with different activities on irradiation induced DNA fragmentation. (A) Agarose gel electrophoresis of pcDNA3.0 exposed to γ-radiation at a dose rate of 1 Gy/min on an ice bath in the presence of heparin-SOD at various enzyme activities. Lanes 1-6 were pcDNA3.0 treated with heparin-SOD with enzyme activity of 0, 7.5×10^2, 1.5×10^3, 3.0×10^3, 6×10^3, 1.2×10^4 units, respectively. **(B)** Analysis of pcDNA3 agarose gel electrophoresis density integral calculus. Open columns, SC, supercoiled DNA; Closed columns, OC, open circular DNA.

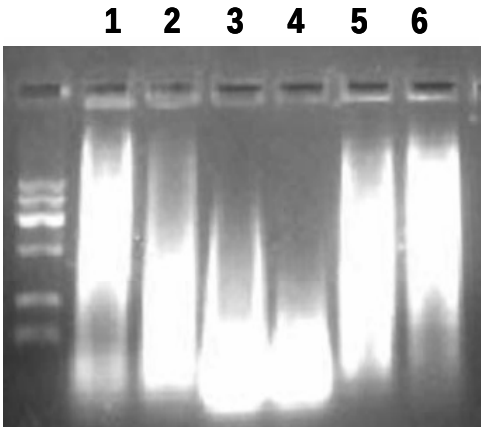

Figure 3. Agarose gel electrophoresis of thymus DNA fragmentation from mice intraperitoneal injection of SOD 40 min before exposure to γ-radiation at a dose rate of 0.30 Gy/min. Lane 1, control (received intraperitoneal injection of 0.5 mL of isotonic sodium chloride 40 min before exposure to γ-radiation); Lane 2, intraperitoneal injection of SOD 40 min before exposure to γ-radiation; Lane 3, intraperitoneal injection of SOD + heparin 40 min before exposure to γ-radiation; Lane 4, intraperitoneal injection heparin-SOD 40 min before exposure to γ-radiation; Lanes 5 and 6, no irradiation exposure.

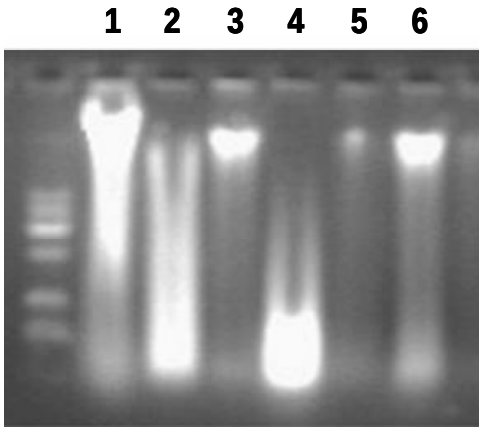

Figure 4. Agarose gel electrophoresis of thymus DNA fragmentation of mice injected with heparin-SOD 3 min after exposure to γ-radiation at a dose rate of 0.30 Gy/min. Lane 1, control (no radiation administered); Lane 2, isotonic Na chloride group; Lane 3, intraperitoneal injection of heparin-SOD 3 min after exposure to γ-radiation; Lane 4, intraperitoneal injection of heparin-SOD 40 min before exposure to γ-radiation; Lanes 5 and 6, tumor injection of heparin-SOD 40 min before exposure to γ-radiation.

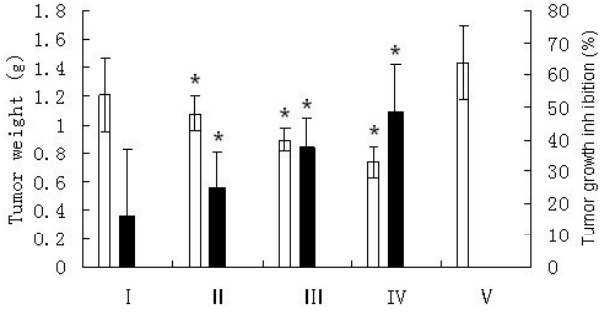

Figure 5. Tumor growth inhibition rates of irradiated S180 sarcoma bearing mice treated with different SODs. Inhibition rate of tumor growth = (tumor weight of untreated group – tumor weight of treated group)/tumor weight of treated group × 100%. I, control (isotonic sodium chloride); II, SOD; III, heparin + SOD; IV, heparin-SOD; V, no irradiation exposure. (I ~ IV: received intraperitoneal injection 40 min before exposure to γ-radiation). * Compared with control group, the difference was considered significant, $p < 0.05$. Open columns, tumor weight; Closed columns, tumor growth inhibition rates.

3.3. *Influence of heparin-SOD on the irradiation effect on tumor growth*

As shown in Figure 5, the tumor inhibition rate of heparin-SOD or heparin + SOD group was significantly higher than that of the control group, and the inhibition rate of the heparin-SOD group was the highest.

4. Discussion

The use of ionizing radiation has become an integral part of modern medicine. In some cases, radiation may be the single best treatment for cancer. Cancer radiation therapy depends on achieving a therapeutic differentiation between cancer cell toxicity and normal tissue toxicity. Therapeutic differentiation may be achieved with chemical radiation sensitizers or protectors (*14,15*). The development of radiation protectors is important not only to enhance the effectiveness of cancer treatment, but also for studying the underlying mechanisms of radiation cytotoxicity (*16*). A wide variety of compounds have been tested. These compounds, though highly effective in *in vitro* studies, may find little use in clinical applications. SODs are one class of essential enzymes in the cellular defense system against the superoxide anion radical ($O_2^{\bullet -}$). Although the importance of SODs as antioxidant enzymes has been demonstrated in various types of cells, whether SODs contribute to the cell's protection against ionizing radiation is still under debate. There are conflicting reports concerning the correlation between the effects of SOD and cellular damage when exposed to ionizing radiation (*17-20*). In the present study, we found that SOD and heparin-SOD rendered protection against γ-radiation induced DNA damage *in vitro* (Figures 1 and 2), but increased sensitivity to ionizing radiation with *in vivo* administered pre-irradiation (Figures 3 and 4).

It is well known that reactive oxygen species (ROS) cause DNA damage and induce cytotoxicity. They induce a variety of lesions in DNA, including oxidized bases, abasic sites, DNA strand-breaks, and cross-links between DNA and proteins (*11,12*). Increase in DNA damage after γ-irradiation has been observed in different studies (*11-13*). DNA constitutes the primary vital target for cellular inactivation of living systems by ionizing radiation. Ionizing radiation-induced damages to cellular DNA are mainly strand breaks of the double- and single-strand types, base damage, elimination of bases, and sugar damage (*21*). The majority of the free radicals may react with DNA by adding to the double bonds

of the bases, forming base radicals, leading to strand breaks (*21*). In this study, the effect of heparin-SOD on the protection of γ-radiation inducted strand breaks in pcDNA3.0 *in vitro* was monitored using agarose gel electrophoresis and observing the disappearance of the SC form of DNA. The reduction in the quantity of the SC form of plasmid DNA was directly related to the radiation-induced damage of DNA. It was found that when pcDNA3.0 was exposed to γ-radiation, the SC form of the molecule was converted to OC form. The presence of heparin-SOD or SOD along with DNA during irradiation prevented this decrease of the SC form, as evidence of attenuating strand breaks. Among all the groups above, the degree of pcDNA3.0 injury when mixed with heparin-SOD was the lowest. It also showed that the protective effect increased with an increase in heparin-SOD concentration (Figures 2A and 2B). Our results revealed that heparin-SOD could effectively protect plasmid DNA against ionizing radiation in an *in vitro* system independent of DNA repair and other cellular defense mechanisms.

Interestingly, the *in vivo* study showed contradictory results for thymus DNA fragmentation when SOD, heparin-SOD, or heparin + SOD was administered pre- and post-irradiation (Figures 3 and 4). When heparin-SOD, SOD, or heparin + SOD was administered pre-irradiation, agarose gel eletrophoresis (Figures 3 and 4) showed that thymus DNA injury was more serious than that of the control group, and when heparin-SOD was administered 3 min shortly after whole body irradiation almost no thymus DNA damage was found (Figure 4, lane 3). The former result was coincident with the early report that overexpression of SOD in *E. coli* had been found to increase sensitivity to ionizing radiation (*22,23*), but contradicted our above *in vitro* experiments (Figures 1 and 2).

The DNA damaging effect of SODs administered pre-irradiation may be explained by the increased level of H_2O_2 in the tissue. It is well known that high levels of H_2O_2 lead to DNA damage. It was reported that human and mouse cell clones overexpressing human Cu,Zn-SOD appeared to have higher levels of H_2O_2 (*24-26*). Other research showed that in some conditions increased amounts of SOD indeed caused increased steady-state levels of H_2O_2 (*27*). In JB6 cells and Chinese hamster fibroblasts, overexpression of Cu,Zn-SOD resulted in increased DNA breakage upon exposure to oxidants (*25,28*). Zhong *et al.* (*29*) reported that overexpression of SOD in rat glioma cells not only inhibited cell growth but also resulted in sensitization to oxidative damage. Researches by Han *et al.* and Wang *et al.* clearly demonstrated that Cu,Zn-SOD could mediate significant DNA cleavage in the presence of either H_2O_2 or mercaptoethanol (*30,31*) implying that any form of the copper-containing SOD enzymes (including Cu,Zn-SOD and its mutants) might have DNA cleavage activity (*32*). In theory, irradiation causes high levels of $O_2^{\bullet -}$, and high levels of $O_2^{\bullet -}$ will produce high levels of H_2O_2 at high levels of SOD. In this study, DNA injury of the intraperitoneal injection group of heparin-SOD before irradiation was the most serious (Figure 4), suggesting that heparin-SOD could serve as a sensitizer in tumor radiotherapy. The contradictory effects of SODs in *in vitro* and *in vivo* experiments imply that irradiation protection or damage is a complex issue and many factors may be involved in the process.

The DNA protecting or repairing effect of heparin-SOD administered post-irradiation is hard to explain. We speculated that when heparin-SOD was absorbed after intraperitoneal injection, the level of $O_2^{\bullet -}$ has become normal or lower, and at this time heparin-SOD does not help to produce a high level of H_2O_2, but helps to recover the activity of DNA repair enzymes. Recently, research indicated that low-molecular-weight heparin could exert beneficial effects on biological macromolecules, such as DNA (*33*). Therefore, we speculated that under some conditions heparin-SOD has DNA repair enzyme activity. In considering the less DNA fragmentation in the thymus in the group which received intratumoral injection of heparin-SOD 40 min before exposure to γ-radiation (lane 6 in Figure 4), we speculate that the intratumoral injection of heparin-SOD was hard to be absorbed into the blood stream due to the solidness of the tumor. The delayed absorption of heparin-SOD acts just like administration of SOD post-irradiation. All these speculations need to be confirmed by experiments. Regardless of the reason, the result suggests that heparin-SOD can be used to treat radiation damage.

Many other studies have clearly demonstrated that SOD suppressed cell growth (*34-44*). SOD had been found to be low in many cancer cells (*34-40*), fetal cells (*41-43*), as well as stem cells (*44*). Forced overexpression of Mn-SOD slowed the growth of cancer cells both *in vitro* and *in vivo* (*41,44*). This growth suppression could be in part a result of increasing the flux of H_2O_2 and thereby pushing the redox status of the cell to a more oxidized state (*43*). The results of our research (Figure 5) showed that the tumor weights of heparin-SOD, SOD, or heparin + SOD groups were lower than that of the control group, and the inhibition rate of the heparin-SOD group was the highest group, suggesting that the use of heparin-SOD could increase the tumor radiotherapy effect.

In summary, the results of our work probably have a meaning to direct selecting a drug administration time when SOD or its modified analogues can be used to prevent or treat radiation injury during tumor radiotherapy, at the same time, a new subject is raised that SOD and its modified analogues may be used to increase tumor sensitivity to radiotherapy.

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