

# Preliminary study of the toxicity and radioprotective effects of zymosan *in vitro* and *in vivo*

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## Research article

**Keywords:** Zymosan, radioprotection, cytotoxicity, apoptosis, survival rate

**Posted Date:** October 12th, 2020

**DOI:** <https://doi.org/10.21203/rs.3.rs-37397/v3>

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**Version of Record:** A version of this preprint was published at BMC Pharmacology and Toxicology on March 17th, 2021. See the published version at <https://doi.org/10.1186/s40360-021-00482-1>.

## Abstract

**Background:** This study aimed to confirm the cytotoxicity of zymosan *in vitro* and *in vivo* and determine the appropriate treatment time and dose of zymosan.

**Methods:** AHH-1 cells and HIECs were administered 0, 20, 40, 80 or 160 µg/mL zymosan. The CCK-8 assay and flow cytometry were used to evaluate cell viability and apoptosis 24 h, 48 h, and 72 h after administration. Furthermore, 12 h before irradiation, the cells were treated with 0, 5, 10, or 20 µg/mL zymosan and then irradiated with 4 Gy X-rays. Cell viability and apoptosis were measured by the CCK-8 assay and flow cytometry at 24 h. In addition, the protective effect of zymosan against radiation *in vitro* was compared to that of 20 µg/mL LPS. *In vivo*, weight, the spleen index and the thymus index were measured to evaluate the toxicity of 0, 5, 10, 20 and 10 mg/kg zymosan. In addition, rats were treated with 0, 2, 4, 8 or 10 mg/kg zymosan and then irradiated with 7 Gy X-rays. The survival rate, organ index were evaluated. The protective effect of zymosan against radiation *in vivo* was compared to that of 10 mg/kg LPS a positive control.

**Results:** The viability and apoptosis of cells treated with different doses of zymosan for different treatment times were not different from those of control cells ( $p<0.05$ ). Furthermore, cell viability and apoptosis were clearly improved after zymosan preadministration ( $p<0.05$ ). The radioprotective effect of zymosan was dose-dependent. In addition, the viability of cells pretreated with zymosan was higher than that of cells pretreated with LPS, and the apoptosis rate of zymosan-treated cells was lower than that of cells pretreated with LPS ( $p<0.05$ ). *In vivo*, weight, the spleen index and the thymus index were significantly decreased by zymosan at a concentration of 20 mg/kg ( $p<0.05$ ). Further experiments showed that the concentration at which zymosan exerted radioprotective effects was 10 mg/kg. The radioprotective effect of zymosan was better than that of LPS pretreatment ( $p<0.05$ ).

**Conclusion:** Zymosan administration before radiation exposure significantly increased cell viability and the survival rates of rats.

## Background

Humans are inevitably exposed to some radiation derived from various trace radionuclides, such as cosmic rays [1], buildings [2], and necessary daily activities [3], but these natural sources of radiation rarely cause fatal radiation damage to human beings. However, the development and utilization of artificial radiation sources such as nuclear power stations, nuclear reactors, and nuclear weapons have forced mankind to face new life-threatening risks [4]. Safety issues with nuclear sources can result in the generation of ionizing radiation, which may cause fatal radiation damage to humans or other organisms [5]. In recent years, increasing attention has been paid to radiation-related research worldwide. At present, the best antiradiation drug approved by the FDA is WR-2721, which is used by the US Army [6]. However, due to its obvious side effects of nausea and vomiting, the use of WR-2721 is restricted to a certain extent [7]. Most radioprotective drugs and drugs used for the treatment of radiation exposure currently in

the research stage have shortcomings such as unclear effects, unclear mechanisms or high toxicity and are limited to preventive administration. When they are injected after radiation, they have no clear treatment effect.

Zymosan, a water-soluble polysaccharide, is mainly prepared from the fungal wall of yeast (*Saccharomyces cerevisiae*). It contains a type of dextran linked by  $\beta$ -1,3 glycoside bonds, and can bind to toll-like receptor (TLR)2 on inflammatory cells [8, 9]. As a TLR-2 agonist, zymosan provided against radiation induced hematopoietic system damage [10]. In addition, zymosan shows a radioprotective effect, inhibiting bone marrow cell apoptosis caused by radiation [11]. Previous studies have found that the radioprotective effects of activated TLR2 and TLR4 are significantly better than those of other TLR ligands and signaling pathways [12, 13]. However, the cytotoxicity of zymosan is unclear, and appropriate treatment time and dose of zymosan are needed to further analyze.

In this study, we analyzed the cytotoxicity of zymosan *in vitro* and *in vivo* and determined the appropriate treatment time and dose of zymosan. The findings provide an experimental basis for the development of safe and effective radioprotective drugs in the future.

## Methods

### *Cell culture*

Human peripheral blood B lymphocytes (AHH-1 cells, BNCC331188) were purchased from BNBIo.com (Beijing, China), and human intestinal epithelial cells (HIECs, MZ-0792) were purchased from Mingzhoubio.com (Zhejiang, China). The cells were cultured in DMEM containing 10% fetal bovine serum at 37 °C and 5% CO<sub>2</sub>.

### *Cell Counting Kit (CCK)-8 assay*

Cells (10<sup>3</sup> cells/well) were cultured in an incubator containing 5% CO<sub>2</sub> at 37°C for 24 hours, and then 10  $\mu$ L of CCK-8 solution (GipBio, Shanghai, China) was added and mixed well. Then, the cells were incubated for 4 h with oscillated and read in a microplate reader. The absorbance of each well at 450 nm was measured by normalization to the blank control. The cell survival rate was calculated based on the following formula: cell survival rate (%) = [(As-Ab)/(Ac-Ab)] × 100.

where As is the absorbance of the experimental well containing cells, medium, CCK-8 and the compounds to be tested, ab = is the absorbance of the blank wells containing medium and CCK-8, and Ac is the absorbance of the control wells containing cells, medium and CCK-8.

### *Flow cytometry*

The Annexin V-FITC/PI Kit (CA1020, Solarbio, Beijing, China) was used to detect cell apoptosis. Cells cultured for 24 hours (1 × 10<sup>6</sup> cells) were collected and washed with precooled phosphate-buffered saline

(PBS). The cells were suspended in 1 mL of 1× binding buffer containing Ca<sup>2+</sup> and centrifuged at 300 ×g for 10 mins. Then, the cell concentration was adjusted to 1×10<sup>6</sup> cells/mL with 1 mL of 1× binding buffer. A total of 100 µL of the cell solution was added to 5 µL of annexin V-FITC and incubated in the dark for 10 min at room temperature. Then, 5 µL of propidium iodide (PI) was added, and the cells were incubated for 5 min. Finally, the volume of the cell suspension was adjusted to 500 µL with PBS and evaluated by flow cytometry (1040, ACEA NovoCyteTM, USA) within one hour. The results were evaluated by Cell Quest software (Version 5.1, BD Biosciences).

### ***Effect of zymosan on cell toxicity***

AHH-1 and HIEC cells were administered 0, 20, 40, 80 or 160 µg/ml zymosan (t1rl-zyn, InvoGen). The CCK-8 assay and cell flow cytometry were used to evaluate cell viability and apoptosis 24 h, 48 h, and 72 h after administration to determine the dose-limiting toxicity of zymosan.

### ***Determination of the dose at which zymosan exerts radioprotective effects on cell***

Twelve hours before irradiation, cells were treated with 0, 5, 10, or 20 µg/ml zymosan and then irradiated with 4 Gy X-rays at a dose rate of 0.25 Gy/min (Synergy, Elekta, Beijing, China). The CCK-8 assay and flow cytometry were utilized to evaluate cell viability and apoptosis at 24 h to determine the optimal dose of zymosan.

### ***Comparison of the radioprotective effects of lipopolysaccharide and zymosan in vitro***

Cells were randomly divided into 4 groups: the normal control (control) group (cells were normally cultured), irradiation only (model) group (cells were irradiated with 4 Gy radiation), LPS group (cells were treated with 20 µg/ml LPS 12 h before being irradiated), and zymosan group (cells were treated with 20 µg/ml zymosan 12 h before being irradiated).

### ***Animals***

A total of 120 male Sprague Dawley rats weighing 180 ± 20 g (aged 6-8 weeks) were purchased from Jinan Pengyue Experimental Animal Co., Ltd. (scxk (Lu) 20190003). The rats were housed at normal temperature (22 ± 2 °C) and humidity (55 ± 5%). Food and water were freely provided, and the animals were housed under a 12-hour light/dark cycle. The rats were adaptively fed for 1 week. The animal experiments were conducted in accordance with the guidelines of the National Institutes of Health (NIH pub. No. 85-23, revised 1996) and were approved by the Animal Protection and Use Committee of Binzhou Medical University.

### ***Toxic effects of zymosan on rats***

The rats were intraperitoneally injected with 0, 5, 10, 20, or 40 mg/kg zymosan, which was dissolved in normal saline. Then, the rats were fed routinely and observed for 21 days. Weight changes were observed weekly. After 21 days, the rats were euthanized with 3% pentobarbital sodium (150 mg/kg). The spleen,

thymus and liver were removed, and the organ index was calculated by the following formula: Organ index = organ weight/body weight [14].

#### ***Determination of the appropriate dose of zymosan for radioprotection of rats***

Twenty-four hours before irradiation, the rats were intraperitoneally injected with 0, 2, 4, 8 or 10 mg/kg zymosan and then irradiated with 7 Gy X-ray at a dose rate of 1.0 Gy/min. Then, they were fed routinely and observed for 21 days. The survival of the rats was assessed each day. After 21 days, the rats were euthanized. The spleen index and thymus index were calculated.

#### ***Comparison of the radioprotective effects of lipopolysaccharide and zymosan in vivo***

Twenty-four rats were randomly divided into 4 groups: the normal control (control) group (the rats were normally fed), irradiation only (model) group (the rats were irradiated with 7 Gy X-ray at a dose rate of 1.0 Gy/min), LPS group (the rats were intraperitoneally injected with 10 mg/kg LPS 24 h before being irradiated), and zymosan group (the rats were intraperitoneally injected with 10 mg/kg zymosan 12 h before being irradiated).

#### ***Statistical analysis***

Prism 5.01 statistical analysis software was used for data processing, and the results are expressed as the mean  $\pm$  standard deviation ( $\bar{x} \pm SD$ ). Data from multiple groups were assessed by one-way analysis of variance (ANOVA), and Tukey's test was used for subsequent analysis.  $p < 0.05$  indicated a significant difference.

## **Results**

#### ***Effect of zymosan on cell viability***

To clarify the effect of zymosan on cell viability, AHH-1 cells and HIECs were treated with different concentrations of zymosan (0, 20, 40, 80 or 160  $\mu$ g/mL). As demonstrated in Figure 1A, the viability of AHH-1 cells was not affected upon treatment with different concentrations of zymosan. The viability of the AHH-1 cells was not affected at 24, 48 or 72 h. Similarly, the viability of the HIECs was not affected upon treatment with different concentrations of zymosan for different time periods (Figure 1B). These results showed that zymosan did not affect cell viability.

#### ***Effect of zymosan on cell apoptosis***

To further confirm that zymosan is not toxic to cells, we evaluated the apoptosis of AHH-1 and HIEC cells. Figure 2A shows the apoptosis rates of AHH-1 cells treated with 20, 40, 80 or 160  $\mu$ g/mL zymosan for 24, 48, or 72 h. There were no clear differences among the different groups of cells ( $p > 0.05$ ). The apoptosis rates of HIECs are shown in Figure 2B. Similarly, the apoptosis rate of HIECs was not affected upon

treatment with different concentrations of zymosan for different time periods. The apoptosis results are shown in the supplementary file. These results indicated that zymosan did not promote apoptosis.

### ***The appropriate dose of zymosan for radioprotection of cells***

The radioprotective effects of zymosan pretreatment on AHH-1 cells and HIECs are shown in Figure 3. The cells were administered 0, 5, 10 or 20 µg/mL zymosan 12 h before being irradiated. The viability of cells exposed to 4.0 Gy radiation was obviously decreased compared with that of control cells ( $p<0.05$ , Figure 3A, B), while the apoptosis rate of the irradiated cells was significantly increased ( $p<0.05$ , Figure 3C, D). The protective effect of zymosan on cells was dose-dependent. The higher the concentration of zymosan, the higher the viability of AHH-1 cells and HIECs (Figure 3A, B) and the lower the apoptosis rate of AHH-1 cells and HIECs (Figure 3 C, D). This indicated that zymosan pretreatment effectively reduced radiation-induced damage to cells. Zymosan was used at a concentration of 20 µg/mL for further study.

### ***Comparison of the radioprotective effects of LPS and zymosan on cells***

It was found that zymosan was superior to the same dose (20 µg/mL) of LPS, which was used as a positive control, in preventing radiation damage. As shown in Figure 4A and B, the viability of AHH-1 cells and HIECs significant increased after pretreatment with LPS or zymosan, and the cell viability of the zymosan pretreatment group was higher than that of the LPS pretreatment group ( $p<0.05$ ). The cell apoptosis assay (Figure 4 C, D) revealed that the apoptosis rates of AHH-1 cells and HIECs were significantly reduced after pretreatment with LPS and zymosan and that the apoptosis rate of cells pretreated with zymosan was lower than that of cells pretreated with LPS ( $p<0.05$ ). This showed that zymosan had a better radioprotective effect than LPS *in vitro*.

### ***Effect of zymosan on rat weight and organ indexes***

*In vivo*, we observed the effect of different concentrations of zymosan, specifically, 0, 5, 10, 20, and 40 mg/kg, on rat weight, spleen index, thymus index and liver index. The results showed that there was no significant difference in body weight between rats intraperitoneally injected with 5 or 10 mg/kg zymosan and control rats ( $p>0.05$ , Figure 5A). At a concentration of 20 mg/kg, zymosan significantly decreased the body weights of the rats ( $p < 0.05$ ), and 40 mg/kg zymosan further decreased the body weights of the rats (Figure 5A). The spleen index, thymus index and liver index were analyzed (Figure 5B), and no significant difference in the spleen index or thymus index was found between rats intraperitoneally injected with 5 or 10 mg/kg zymosan and control rats ( $p>0.05$ ). At a concentration of 20 mg/kg, zymosan significantly decreased the spleen index and thymus index of the rats ( $p < 0.05$ ), and 40 mg/kg zymosan further decreased the spleen index and thymus index (Figure 5A). However, there was no significant difference in liver index among groups ( $p > 0.05$ ). These data revealed that high concentration of zymosan was toxic to rats.

### ***Dose at which zymosan exerts radioprotective effects in rats***

The rats were administered 0, 2, 4, 8 or 10 mg/kg zymosan 24 h before being irradiated. The survival rate, spleen index and thymus index of rats exposed to 7.0 Gy radiation were obviously lower than those of control rats ( $p < 0.01$ , Figure 6). The protective effect of zymosan on rats was dose-dependent. The higher the concentration of zymosan, the higher the survival rate, spleen index and thymus index of the rats. At a concentration of 8 mg/kg, zymosan significantly decreased the spleen index and thymus index of the rats ( $p < 0.05$ ). This suggested that zymosan pretreatment effectively reduced radiation-induced damage to rats. Zymosan was used at a concentration of 10 mg/kg for further study.

### ***Comparison of the radioprotective effects of LPS and zymosan in vivo***

*In vivo*, the radioprotective effects of 10 mg/kg zymosan were compared with those of the same dose of LPS as a positive control. As shown in Figure 7, the survival rate, spleen index and thymus index were significantly decreased in zymosan- and LPS-treated rats compared with control rats ( $p < 0.01$ ). LPS and zymosan pretreatment prior to radiation exposure significantly increased the survival rate, spleen index and thymus index. Furthermore, the protective effect of zymosan was better than that of LPS. All data suggested that appropriate concentration of zymosan pretreatment had a good protective effect on radiation.

## **Discussion**

Nuclear radiation injury mainly involves the destruction of genes and biological macromolecular structures and the degeneration and necrosis of tissue cells caused by ionizing radiation, which causes tissue and organ dysfunction [15, 16]. The mechanisms underlying the occurrence and development of radiation injury have been analyzed thoroughly, but no ideal treatment has been identified. At present, there are two main strategies for protecting against ionizing radiation: one is external protection, that is, reducing the damaging effects of rays on the body by using appropriate shielding materials, and the other is internal protection, which involves developing appropriate antiradiation drugs. Antiradiation drugs can exert radioprotective effects before or after radiation exposure [17, 18]. Currently, most of the drugs used for the treatment of radiation-induced damage are western medicines and are associated with adverse reactions. They are mainly used for irradiation prevention and early treatment after irradiation and are not suitable for long-term use; however, repairing the body after radiation damage requires long-term treatment. Therefore, it is imperative to search for antiradiation drugs with high efficiency, low toxicity, and few side effects that can be orally administered and to explore the mechanisms underlying radioprotection.

Recently, some progress towards the research of antiradiation drugs has been made. The potency of sulfur-containing radiopreventive drugs (cysteamine S-phosphate sodium salt, N-acetylcysteine) is generally low [19, 20]. The potency of vitamins and hormones is equivalent to that of sulfur-containing drugs, but their effective doses are thousands of times the physiological concentrations, and the side effects associated with their long-term use are not easy to overcome [21, 22]. Zymosan is a natural polysaccharide  $\beta$ -glucan that is naturally present in the yeast cell wall, exerts strong effects and is not

associated any side effects. Zymosan has been shown to combine with receptors on the surface of immune cells to regulate immune responses and enhance the immune activity of cells [23]. Taghavi et al. also found that zymosan has antitumor activity and plays a positive role in inhibiting the progression of melanoma by regulating the expression of TLR-2, TLR-4 and tumor necrosis factor-a [24]. Although it has been reported that fungal beta glucan and mushroom  $\beta$ -glucan can improve the survival rate of irradiated mice [25, 26], there has been little research on the antiradiation effect of zymosan. In this research, based on cell and animal studies, the radioprotective effect of zymosan on cells and rats irradiated for different time periods was studied, and the appropriate dose of zymosan was preliminarily determined. Zymosan administration before radiation exposure significantly increased cell viability and the survival rates of rats.

Nevertheless, our results showed that zymosan treatment decreased the weight of rats at a concentration of 20 mg/kg or 40 mg/kg. At the same time, the spleen index and thymus index were also clearly decreased at a concentration of 20 mg/kg or 40 mg/kg. The organ index doesn't capture the effects on the organ entirely. Specifically, changes in cell structure or histopathology may help to explain the toxicity of zymosan in the organs. In the further, the pathological observation of organs is necessary to analyze the toxicity of zymosan.

The survival curves in the irradiated rats were barely separated between the LPS treatment and zymosan treatment. Furthermore, LPS was protective in the cell viability after radiation. Interestingly, the cell viability and organs indexes were higher in zymosan treatment than LPS treatment after radiation. What could improve the efficacy of zymosan is a worthy research direction since it exists the toxicity. Combination of more agents may be increase the radiation protection effect, but this is only our speculation. The above results show that zymosan has an effective protective against ionizing radiation, which is simply as a preventative therapy. Is it feasible for those who are exposed to radiation by accidentally or passively ? This question is needed to further confirm. If zymosan treatment plays protection before and after radiation, it would be used for radiation-related workers, such as individuals exposed to medical radiation, nuclear accident personnel and other radiation workers, and that it has potential application value.

However, the magnitude and severity of nuclear radiation's harm to human health also depend on factors such as the type of radiation, radiation dose rate, dose absorbed by the body and individual sensitivity. Different types of nuclear radiation have significant differences in relative biological effects on organisms. Therefore, in the future, we will address the above issues and provide basic biological data for the development of effective radioprotective drugs.

## Conclusions

In summary, zymosan pretreatment significantly increased cell viability and the survival rate of rats, but the high dose of zymosan might be toxic to rats. In the further, the pathological observation of organs is necessary to analyze the toxicity of zymosan. Many problems still need to actively explore, such as how

to improve the efficacy of zymosan as a preventative therapy and what is its function on those who are exposed to radiation.

## Abbreviations

TLR (Toll-like receptor), pattern recognition receptors (PRRs), nuclear factor kappa beta (NF- $\kappa$ B), myeloid differentiation factor 88 (MyD88), interleukin (IL), tumor necrosis factor (TNF), lipopolysaccharide (LPS)

## Declarations

### Ethics approval and consent to participate

This study was approved by the Animal Protection and Use Committee of Binzhou Medical University (No. 20191214-031).

### Consent for publication

Not applicable

### Availability of data and material

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

### Competing interests

The authors have no conflicts of interest to declare.

### Funding

None.

### Authors' contributions

QZL and JJM analyzed and interpreted the western blot data. YZZ and SJG evaluated cell apoptosis and were major contributors to writing the manuscript. HCL performed the cell activity experiments. All authors read and approved the final manuscript.

### Acknowledgments

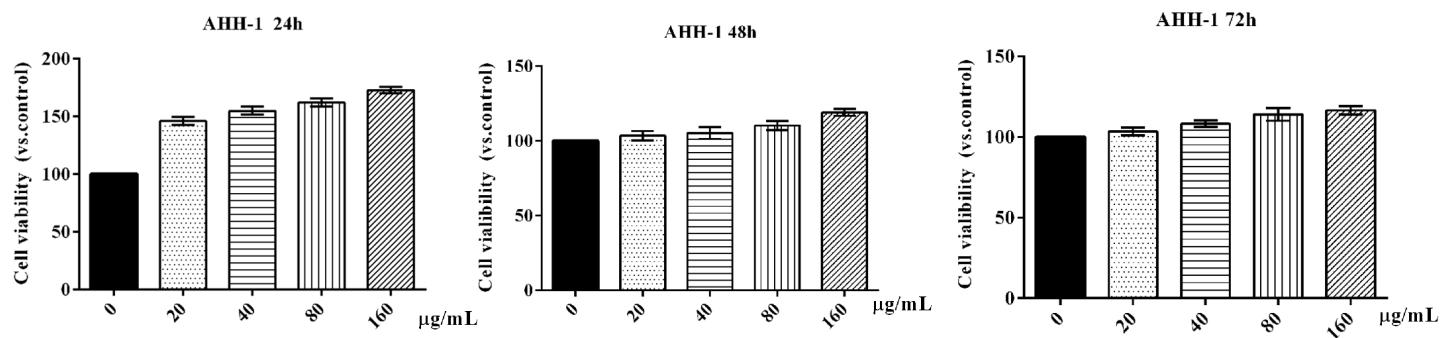
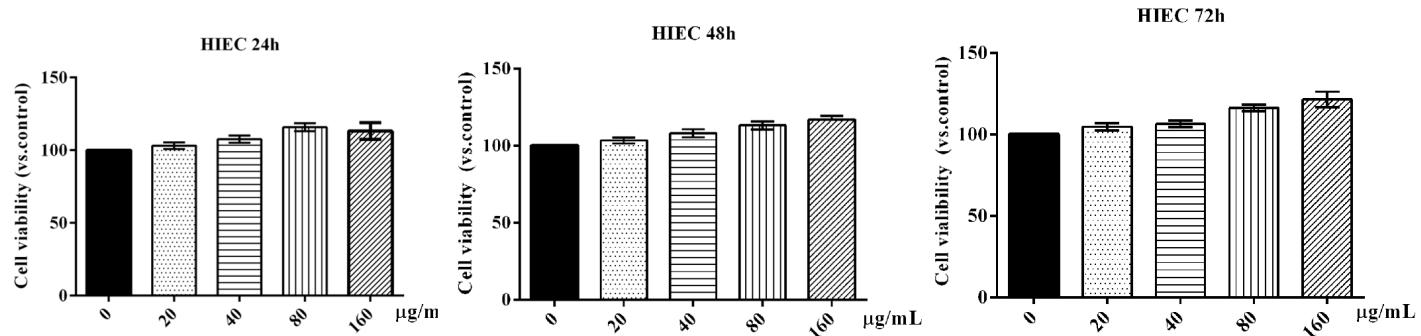
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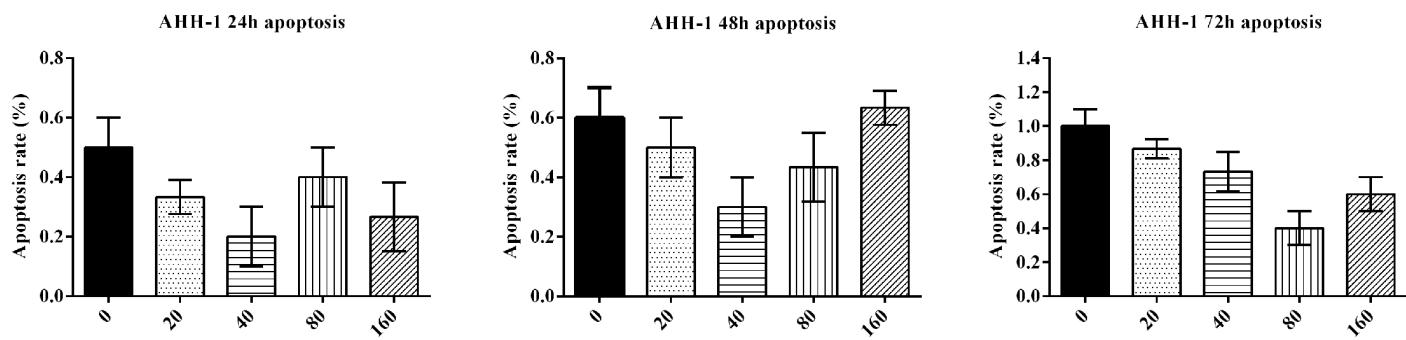
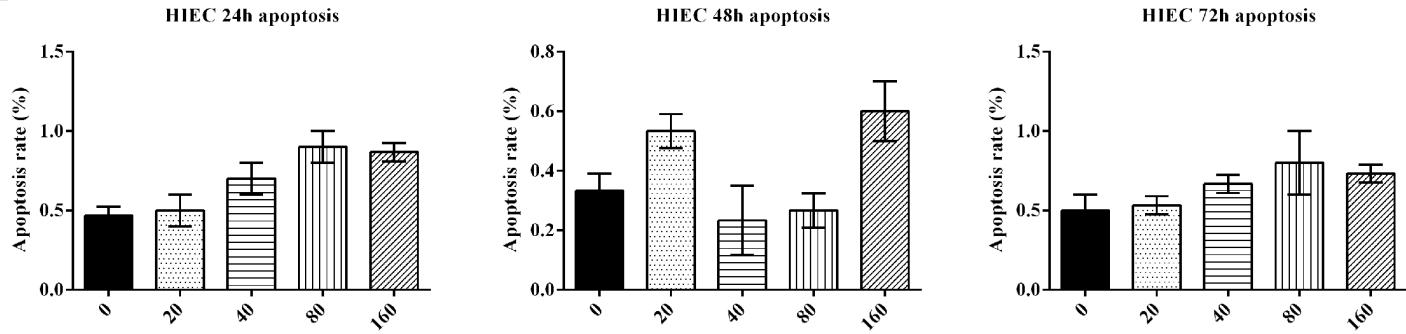
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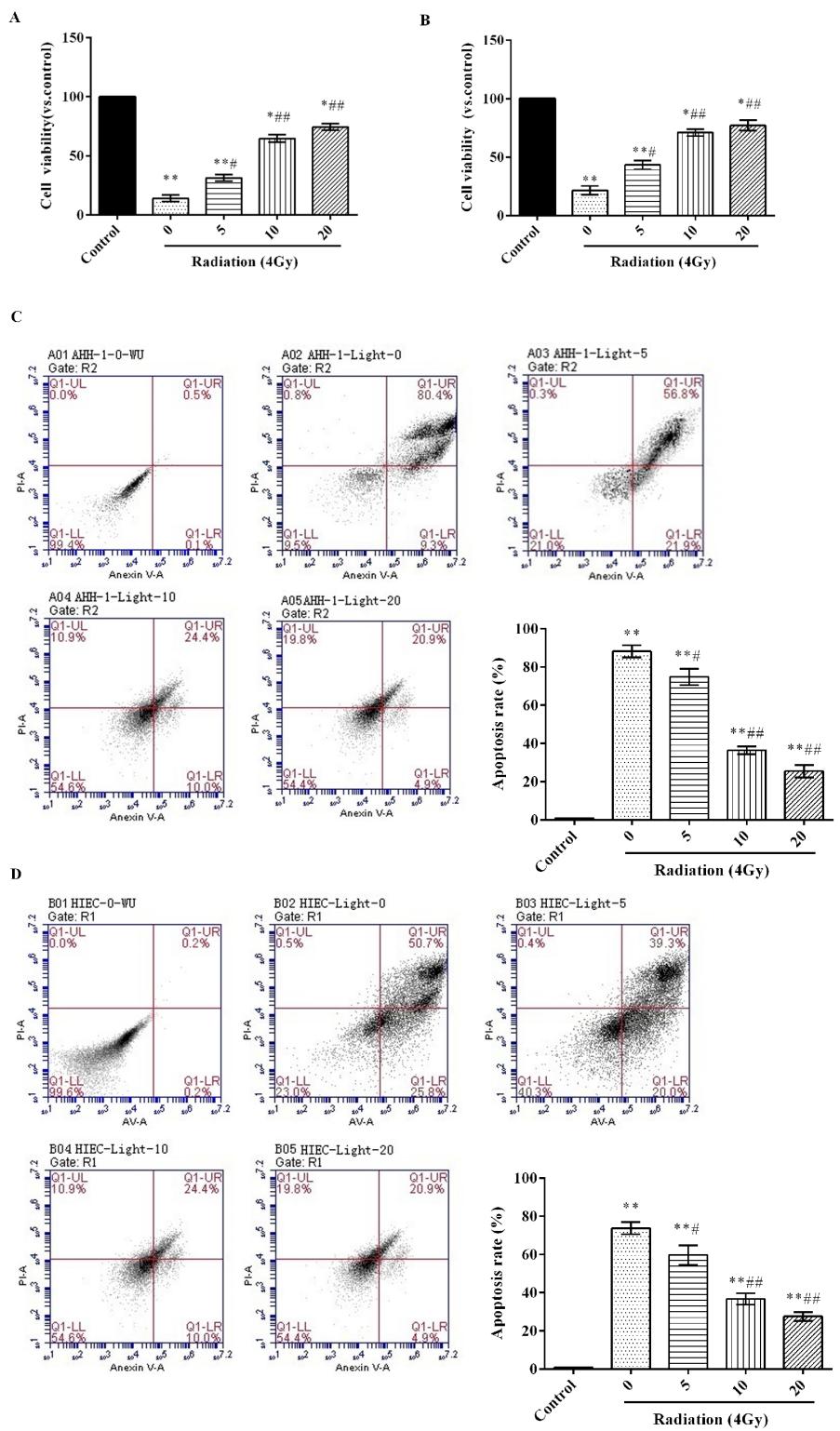
## Figures

**A****B****Figure 1**

The effect of zymosan on cell viability. (A) The viability of AHH-1 cells treated with different concentrations of zymosan (0, 20, 40, 80, or 160  $\mu\text{g/mL}$ ) for 24, 48 or 72 hours. (B) The viability of HIECs treated with different concentrations of zymosan (0, 20, 40, 80, or 160  $\mu\text{g/mL}$ ) for 24, 48 or 72 hours. The test was repeated 3 times. The data are expressed as the  $\bar{x} \pm \text{SD}$ . ANOVA was used to analyze the data from the different groups.

**A****B****Figure 2**

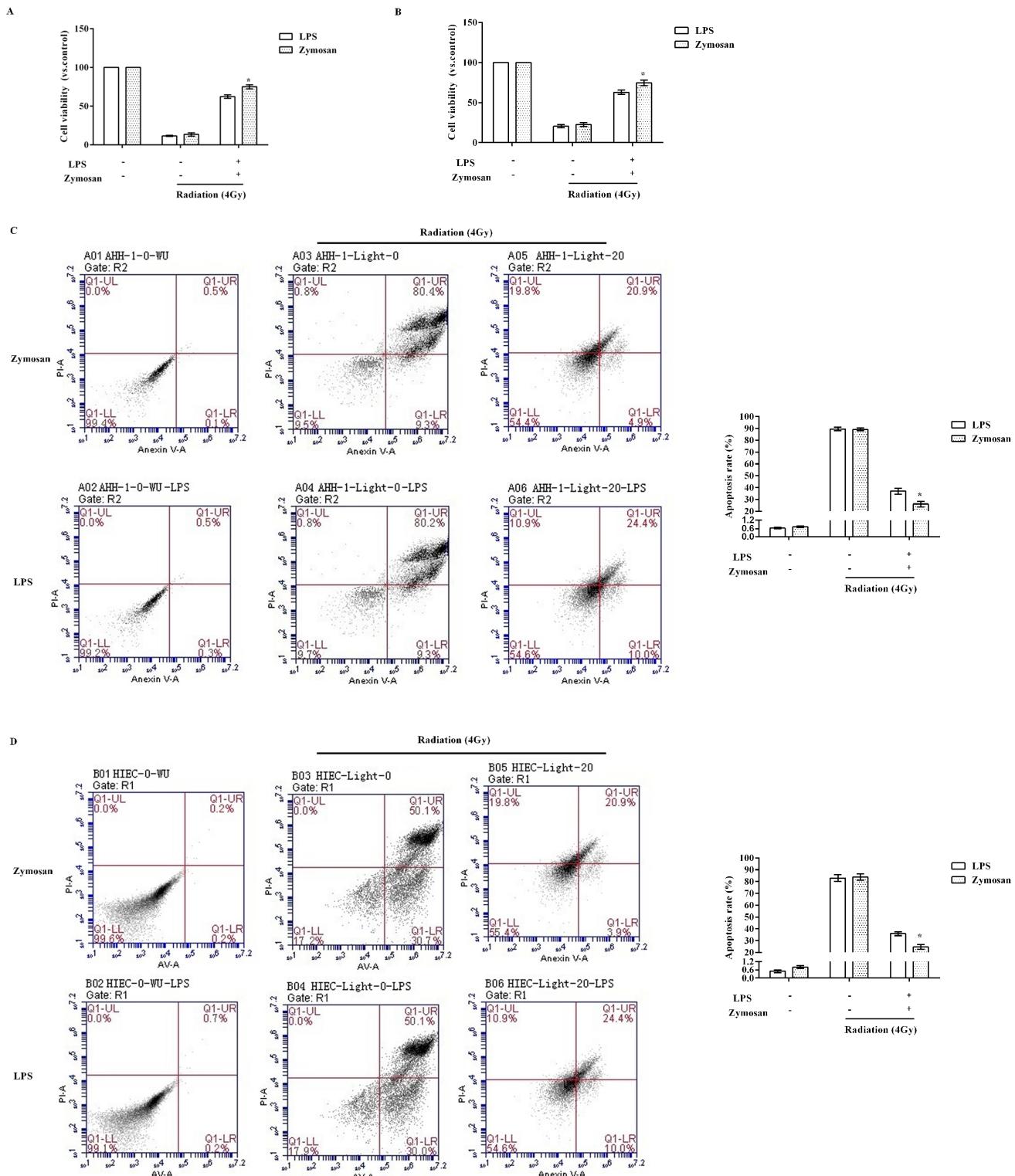
The effect of zymosan on cell apoptosis. (A) The apoptosis rate of AHH-1 cells treated with different concentrations of zymosan (0, 20, 40, 80, or 160  $\mu\text{g/mL}$ ) for 24 h, 48 h or 72 h. (B) The apoptosis rate of HIECs treated with different concentrations of zymosan (0, 20, 40, 80, or 160  $\mu\text{g/mL}$ ) for 24 h, 48 h or 72 h. The test was repeated 3 times. The data are expressed as the  $\bar{x} \pm \text{SD}$ . ANOVA was used to analyze the data from the different groups.



**Figure 3**

The radioprotective effects of zymosan on cells. Cells were treated with different concentrations of zymosan (0, 5, 10, or 20 µg/mL) 12 h before being treated with 4 Gy radiation. (A) The viability of AHH-1 cells after 24 h. (B) The viability of HIECs after 24 h. (C) The apoptosis rate of AHH-1 cells after 24 h. (D) The apoptosis rate of HIECs after 24 h. Compared with the control group, \*\*p<0.01; compared with the 0

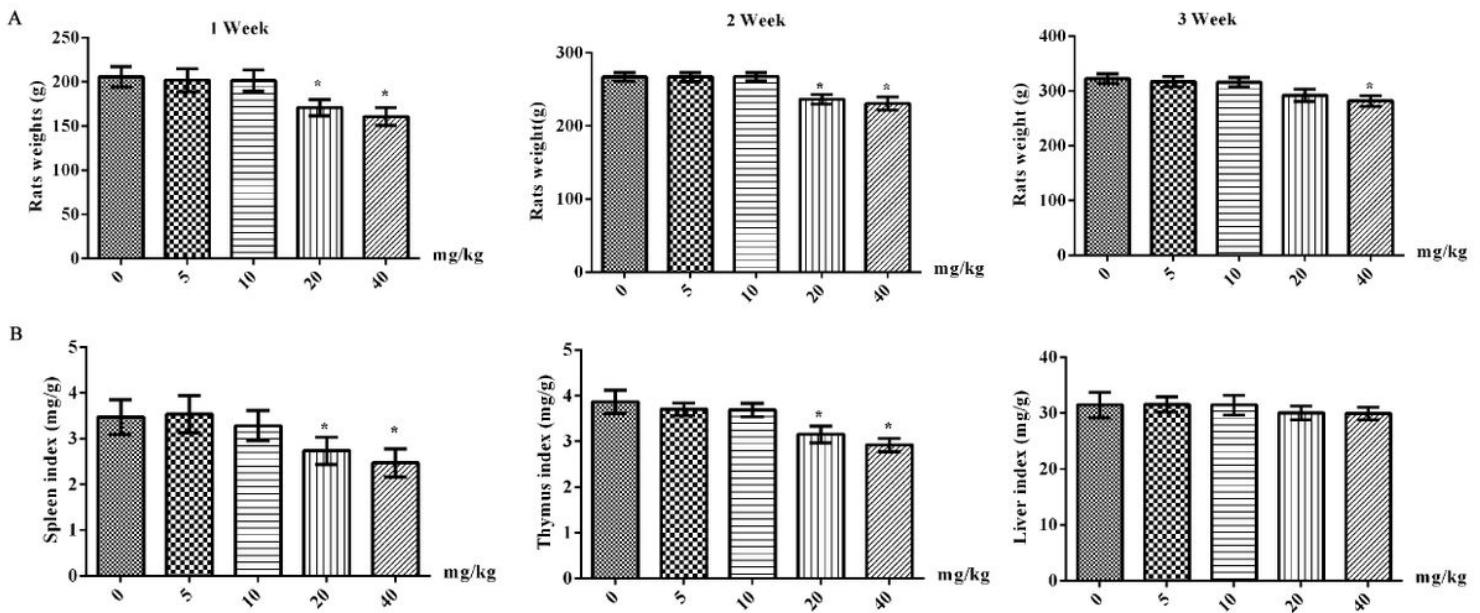
$\mu\text{g/mL}$  zymosan group, # $p<0.05$ , ## $p<0.01$ . The test was repeated 3 times. The data are expressed as the  $\bar{x}\pm\text{SD}$ . ANOVA was used to analyze the data from the different groups.



**Figure 4**

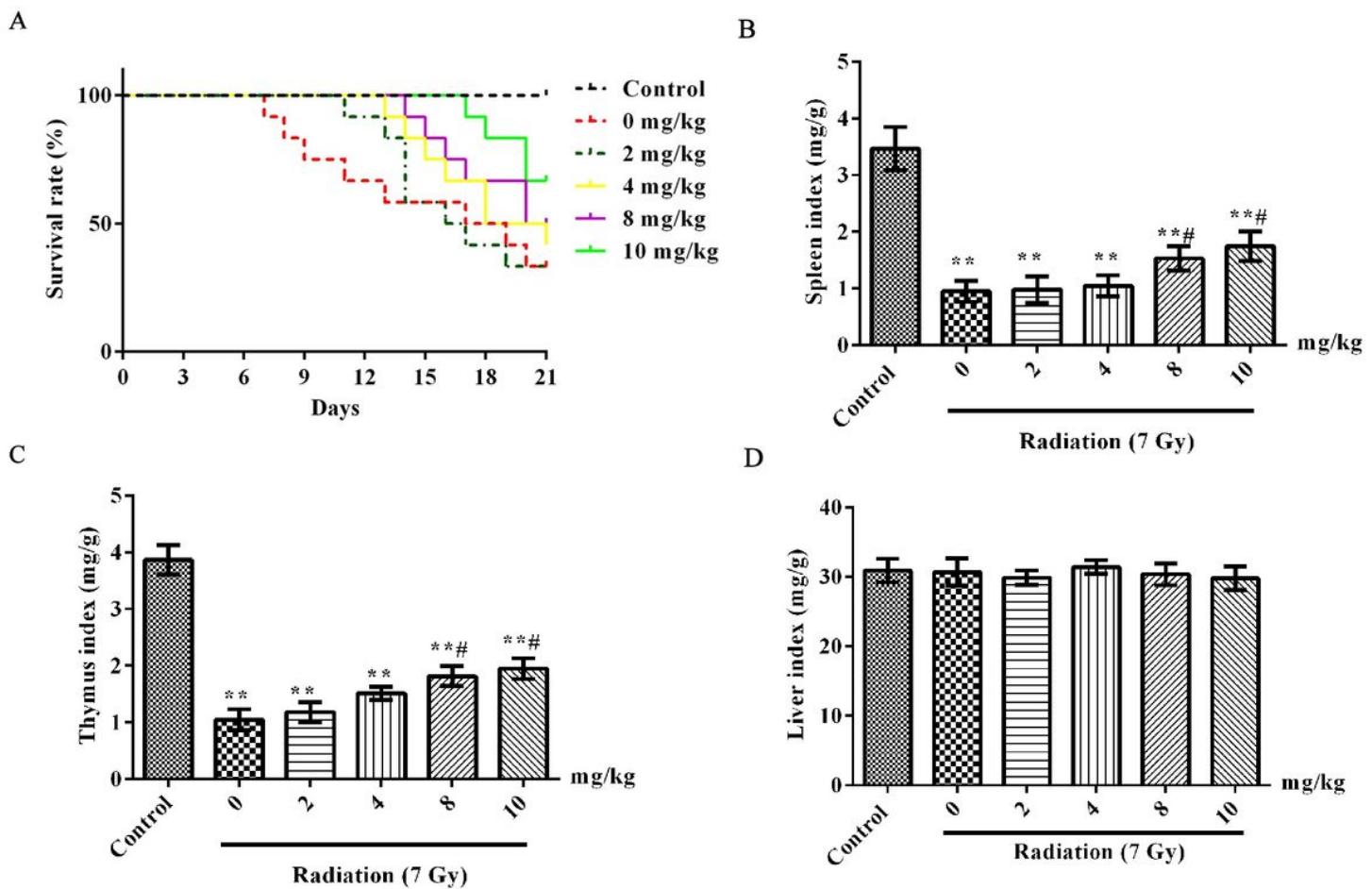
Comparison of the protective effects of LPS and zymosan against radiation in cells. Cells were treated with LPS ( $20 \mu\text{g/mL}$ ) or zymosan ( $20 \mu\text{g/mL}$ ) 12 h before being treated with 4 Gy radiation. (A) The viability of AHH-1 cells after 24 h. (B) The viability of HIECs after 24 h. (C) The apoptosis rate of AHH-1

cells after 24 h. (D) The apoptosis rate of HIECs after 24 h. Compared with the LPS group, \* $p<0.05$ . The test was repeated 3 times. The data are expressed as the  $\bar{x}\pm SD$ . ANOVA was used to analyze the data from the different groups.



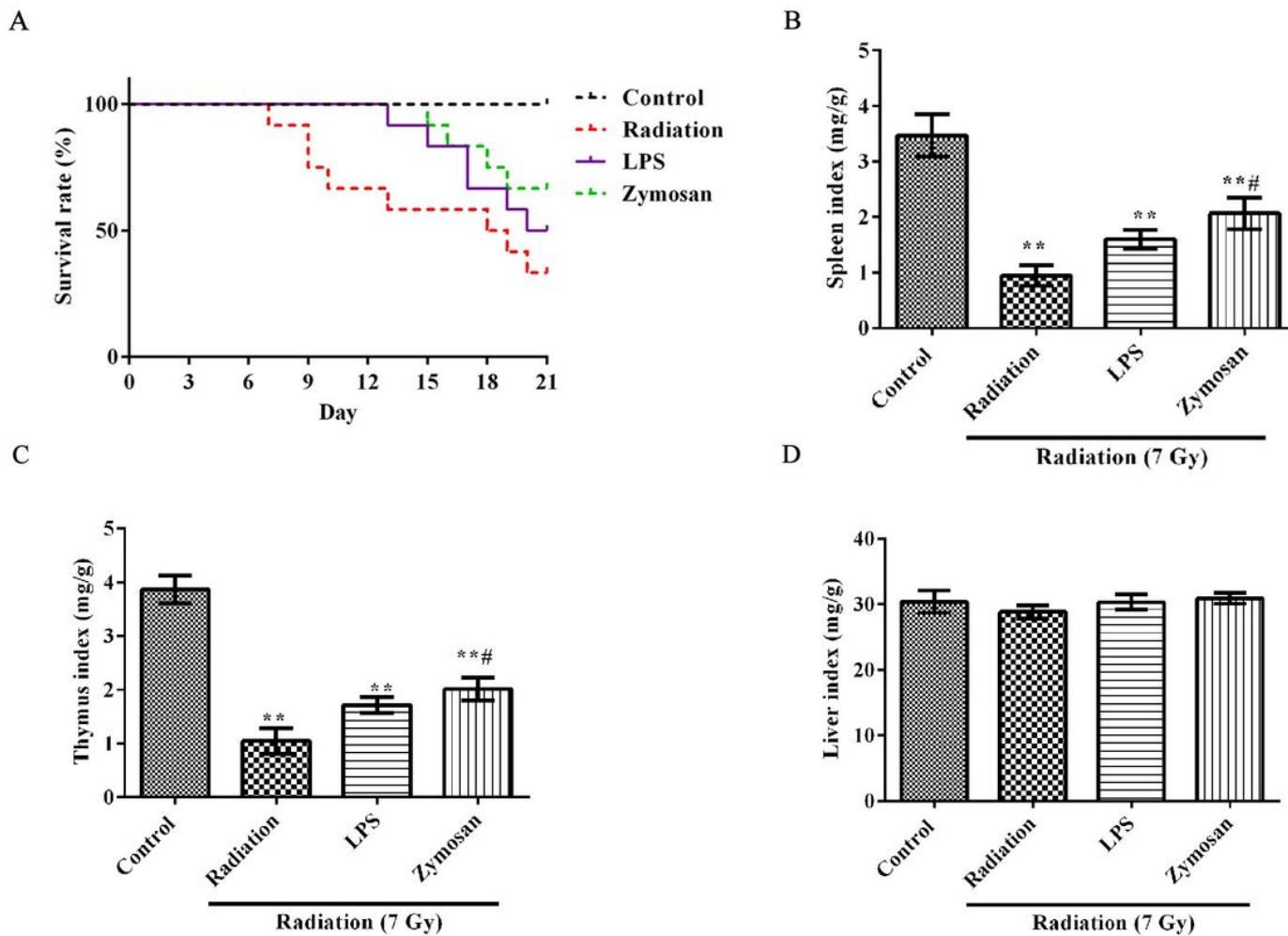
**Figure 5**

The effect of zymosan on rat weight, organs index. (A) The body weights of rats treated with different concentrations of zymosan (0, 5, 10, 20, or 40 mg/kg) for 1 week, 2 weeks or 3 weeks. (B) The spleen index, thymus index and liver index after treatment with different concentrations of zymosan (0, 5, 10, 20, or 40 mg/kg). Compared with the 0 mg/kg zymosan group, \* $p<0.05$ . There were 6 rats in each group. The data are expressed as the  $\bar{x}\pm SD$ . ANOVA was used to analyze the data from the different groups.



**Figure 6**

The radioprotective effects of zymosan in rats. Rats were treated with different concentrations of zymosan (0, 2, 4, 8, or 10 mg/kg) 24 h before being treated with 7.0 Gy irradiation. (A) The survival rate of rats after 21 d. (B) The spleen index; (C) The thymus index; (D) The liver index. Compared with the control group, \*\* $p<0.01$ ; Compared with the 0 mg/kg zymosan group, # $p<0.05$ . There were 12 rats in each group. The data are expressed as the  $\bar{x}\pm SD$ . ANOVA was used to analyze the data from the different groups.



**Figure 7**

Comparison of the protective effects of LPS and zymosan against radiation in rats. Rats were treated with LPS (10 mg/kg) or zymosan (10 mg/kg) 24 h before being treated with 7.0 Gy irradiation. (A) The survival rate of rats after 21 d. (B) The spleen index; (C) The thymus index; (D) The liver index. Compared with the control group, \*\* $p<0.01$ ; Compared with the LPS-treated group, # $p<0.05$ . There were 12 rats in each group. The data are expressed as the  $\bar{x}\pm SD$ . ANOVA was used to analyze the data from the different groups.

## Supplementary Files

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- [Supplementarydocument.docx](#)