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Lycium barbarum polysaccharide inhibits the mitochondrial pathway of apoptosis in mouse bone marrow mononuclear cells after radiation injury

H. Huang[1], Y. Wang[2], L. Zhang[3], F.Q. Hu[3], J. Li[3], H. Pang[3*]

[1]Department of Radiology, Fuling Central Hospital of Chongqing City, Chongqing, 408099, China
[2]Ultrasound diagnostic department, Hubei Maternal and Children Health Hospital, Wuhan, 430070, China
[3]Department of Nuclear Medicine, The First Affiliated Hospital of Chongqing Medical University, Chongqing, 400016, China

► ***Original article***

Corresponding author:
Hua Pang, Ph.D.,
E-mail: *phua1973@163.com*

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ABSTRACT

Background: Lycium barbarum is a traditional Chinese medicine. Its pharmacological effects mainly rely on a component called Lycium barbarum polysaccharide (LBP). The present study aims to explore the mechanism by which LBP reduces radiation damage in X-ray-irradiated cultured mouse bone marrow mononuclear cells （BMNCs） and the potential involvement of apoptosis. ***Materials and Methods:*** Mouse BMNCs were cultured in vitro and exposed to radiation. After 24 hours of LBP treatment, BMNCs viability was detected by cck-8 method, apoptosis rate was examined by Flow cytometry (FCM), Mitochondrial membrane potential (MMP) fluorescence was detected by JC-1, and the expression of mitochondrial pathway-associated protein was measured by immunoblotting. ***Results:*** LBP significantly increased BMNCs viability and reduced the apoptosis rate of radiation-exposed BMNCs 24 h after treatment compared to the non-treated control group. In a mitochondrial membrane potential fluorescence assay, LBP reduced the radiation-induced decrease in mitochondrial membrane potential. Western blot analysis further proved this point, where LBP inhibited the release of cytochrome C from mitochondria and also inhibited the expression of caspase 9 and other mitochondrial-related proteins. ***Conclusions:*** The mechanism of LBP action in radiation-exposed mouse BMNCs cells seems to involve inhibition of the mitochondrial apoptosis pathway.

INTRODUCTION

Ionizing radiation is widely used in medical diagnosis and treatment. In radiation therapy, while the goal is to destroy the tumor tissue, the normal tissues must be protected at the same time. Radiation not only damages DNA directly, but also leads to the generation of excess free radicals and induces the activation of various signal molecules and pathway [1, 2, 3]. The result is an alteration of the biological behavior of the cell and, finally, apoptosis.

At present, most radiation-protective drugs are sulfhydryl compounds, cytokines and hormones, but these are highly toxic and expensive [4, 5]. Lycium barbarum polysaccharide (LBP) is an extract of Lycium barbarum, which is mainly composed of a mixture of monosaccharides such as rhamnose, xylose, galactose, and arabinose [6]. It is also an inexpensive Chinese traditional medicine with a very long history of use and positive effects [7] including anti-radiation [8], anti-tumor [9], anti-aging [10], hypoglycemic [11], reproductive protection [12], and immune-regulatory activities effects [13, 14].

Among the strong immunomodulatory effects of LBP is a promotion in phenotypic and functional maturation of murine dendritic cells [15], and a significant improvement in the natural killer (NK) cell activity of human [16]. The anti-tumor activity of LBP includes inducing quiescence by allowing cells to stay in the S phase of the cell cycle and increasing intracellular Ca^{2+} concentration to induce tumor cell apoptosis [17]. LBP prevents, and also ameliorates, the negative effects of ionizing radiation via actions that include an increase in the proliferative activity of bone marrow cells, a reduction in the apoptotic rate, regulation of cyclin expression [18], and elimination of excess free radicals [19]. In the bone marrow, LBP can reduce the apoptosis of bone marrow mononuclear cells (BMNCs) caused by radiation damage and accelerate cell cycle transition [20].

Based on the abovementioned findings, we further investigated the mechanism by which LBP protects radiation-damaged BMNCs and the role of apoptosis. This research aims to explore the effects of LBP on the expression of mitochondrial membrane potential and apoptosis-related proteins, and provide a

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theoretical basis for the development of new radioprotective agents based on LBP and related compounds.

MATERIALS AND METHODS

Reagents

LBP was from Shanghai Kangzhou Fungi Extract Co., Ltd. (Shanghai, China). Mouse BMNC separation kit was purchased from TBD Science (Tianjin, China). Cell Counting Kit-8 (CCK-8) was obtained from Beijing Biodragon Immunotechnologies Co. (China). The annexin V-FITC/PI apoptosis detection kit and mitochondrial membrane potential detection kit (JC-1) were from Beyotime Biotechnology (Shanghai, China). Cytochrome C (Cyt C), poly (adenosine diphosphate ribose) polymerase (PARP), caspase 9, and caspase 3 antibodies for western blot analysis were purchased from Cell Signaling Technology (Beverly, MA, USA), and Cox IV and β-actin antibodies were from Proteintech (Wuhan, China). All other chemicals used in this study were of analytical grade and are commercially available.

Animals

The 30 male Kunming mice aged 1.5 to 2 months used in this study were from the Experimental Animal Center of Chongqing Medical University (Chongqing, China). The complete animal experiment protocol has been reviewed and approved by the institutional ethics committee (ethics approval number: 2017-119). All animal procedures strictly abide by the principles of use and care of laboratory animals.

Cell culture and treatment

Under sterile conditions, the mice were euthanized by using a high concentration of carbon dioxide. The femoral connective tissues and muscles were removed bilaterally. BMNCs in the femur was obtained by washing with F solution (mouse BMNC separation kit; F2013TBD; TBD Science, Tianjin, China) and centrifuging. The sample was diluted using a sample dilution solution (mouse BMNC separation kit; TBD Science) and the resulting cell suspension was slowly transferred to the monocyte separation solution (mouse BMNC separation kit; TBD Science). Centrifuge again, and the sample was divided into four parts from top to bottom. Cells in the second, milky white BMNC layer were transferred to a clean centrifuge tube and washed three times with cleaning solution (mouse BMNC separation kit; TBD Science). Finally, the cells were resuspended in culture medium and cultured in an incubator.

Grouping of cells: blank (no radiation or LBP treatment), control (irradiation only), 200 μg LBP/mL (irradiation + 200 μg LBP/mL), 400 μg LBP/mL (irradiation + 400 μg LBP/mL), and 800 μg LBP/mL (irradiation + 800 μg LBP/mL). X-ray irradiation was carried out at room temperature using a linear accelerator. The dose was 4 Gy, delivered at a rate 300 cGy/min. immediately after irradiation, cells in the LBP groups were transferred to medium containing the appropriate amount of LBP, and those of the blank and control groups to normal medium for further culture. The radiation dose was determined according to preliminary experiments, and the LBP concentration in cytotoxicity experiments (data not shown).

CCK-8 method for detecting cell viability

The cells were seeded in 96-well plates. Then, 24 hours after the LBP treatment, the cells were incubated with 10 μL of CCK-8 solution, and the absorbance (optical density; OD) of each well at 450 nm was measured 2 hours later. Calculate the cell survival rate as the OD value of the experimental group/control group. The same method was repeated three times.

Apoptosis rate

The apoptosis rate of each group was detected 24 h after LBP treatment by flow cytometry (FACS Caliber; BDX, Franklin Lakes, USA) using annexin V/propidium iodide (PI) staining. Annexin V-positive and PI-negative are early apoptosis of cells, while annexin V-positive and PI-positive are cells in the terminal stage of apoptosis or cells have died. .

Mitochondrial membrane potential fluorescence

Mitochondrial membrane potential was determined 24 h after LBP treatment using a JC-1-based fluorescence assay kit (C2006; Beyotime) and fluorescence microscopy (Eclipse Ti-S; Nikon, Tokyo, Japan).

Western blot

The cells in each group were tested for protein expression 24 h after LBP treatment. Mitochondria were isolated from the cells using a cell mitochondrial isolation kit (Beyotime, China), and the protein concentration was confirmed using a BCA protein assay kit (Beyotime, China). First, 30 μg of protein was electrophoretically separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 12% or 8% polyacrylamide gels and the proteins in the gel were subsequently transferred to a poly (vinylidene fluoride) membrane. After blocking, it was incubated overnight at 4°C with Cyt C, caspase 9, caspase 3, PARP, Cox IV, and β-actin antibodies, then washed four times with Tris-buffered saline and Tween-20, and incubated with goat anti-mouse or goat anti-rabbit IgG secondary antibody for 1 h at room temperature. Finally, the chemiluminescence method was used to visualize the bands on the membrane.

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Statistical analysis

The experimental data were processed using SPSS software (ver.22; SPSS Inc., Chicago, USA) and expressed as means ± standard error (SE). ANOVA was used to compare the experimental and control groups, and the Dunnet-t test was applied for comparisons between two experimental groups. The blank and control groups were compared using a t-test. A p value <0.05 was considered to indicate statistical significance.

RESULTS

The effect of LBP on cell viability

As shown in figure 1, cell viability, measured using the CCK-8 method, was higher in the blank group than in the control group ($p < 0.05$), whereas the activity of LBP-treated BMNC cells was significantly higher than that of the control group ($p < 0.01$). The latter result suggested the ability of LBP to promote the activity of irradiated cells, with an optimal dose of 400 μg/mL.

LBP inhibits radiation-induced cell apoptosis

To determine the ability of LBP to inhibit radiation-induced apoptosis, the number of apoptotic cells was analyzed by flow cytometry 24 h after LBP treatment. LBP (400 μg/mL) inhibited radiation-induced damage by reducing the number of apoptotic cells (figure 2A) whereas radiation-induced apoptosis in the control was increased (figure 2B). Compared to the control group, the early apoptotic rate of BMNC cells was significantly lower after LBP treatment ($p<0.01$). A significant decrease in the total apoptosis rate compared to the control was obtained after treatment of the irradiated cells with 400 μg LBP/mL ($p <0.05$). These results indicated that LBP attenuates radiation-induced apoptosis, with optimal results obtained at a dose of 400 μg/mL.

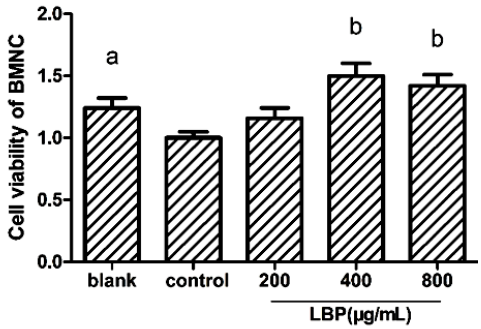
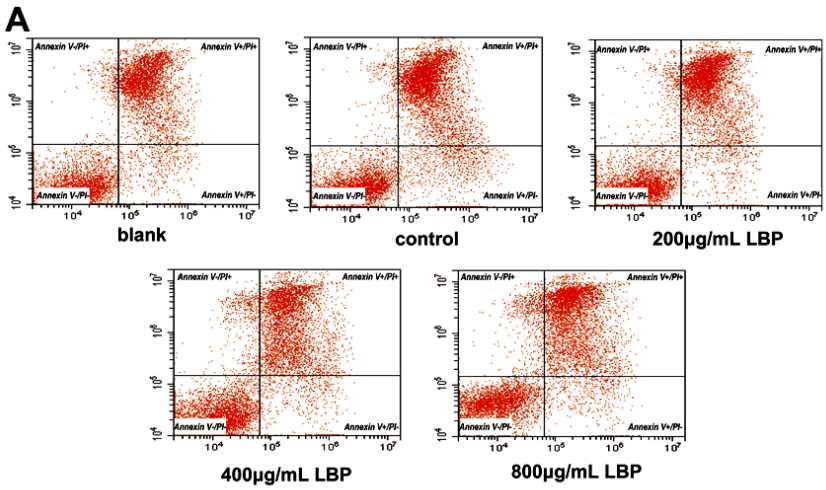


Figure 1. Radiation-induced effects on cell viability 24 h after exposure of bone marrow mononuclear cells (BMNCs) to Lycium barbarum (LBP). Bone marrow mononuclear cells (BMNCs) were exposed to three concentrations of LBP after irradiation; cell viability was then measured by CCK-8 assay. The data are expressed as means ± SE (n = 3). [a]p <0.05 and [b]p <0.01 compared with the control group.



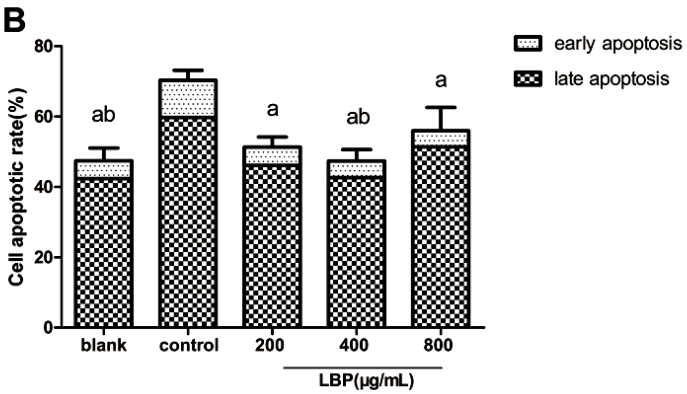


Figure 2. LBP inhibits radiation-induced early and late apoptosis. BMNCs were irradiated with 4 Gy and treated (or not) with LBP. Annexin V/PI staining was used to show the number of apoptotic cells (A). The data are shown as means ± SE (n = 3) (B). [a]p <0.01 compared with the control in assays of early apoptosis; [b]p <0.05 ,compared with the control in assays of total apoptosis.

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Effect of LBP on mitochondrial membrane potential

The effect of LBP on radiation-induced mitochondrial membrane potential was examined based on JC-1 red-green fluorescence, examined using fluorescence microscopy 24 h after LBP treatment. In the irradiated control, the radiation-induced red-green fluorescence ratio was reduced (figure 3A) whereas after LBP treatment it was significantly increased (figure 3B, $p < 0.01$). The results showed the ability of LBP to decrease mitochondrial membrane potential in radiation-exposed cells.

LBP inhibits the expression of mitochondrial pathway-associated proteins

To elucidate the molecular mechanism of LBP in radiation-induced apoptosis, several apoptosis-related proteins were examined by western blotting. As shown in figure 4A, mitochondrial Cyt C expression was decreased after irradiation, whereas cytoplasmic Cyt C expression was increased. LBP treatment promoted mitochondrial Cyt C expression (figure 4B, $p < 0.05$) and inhibited cytoplasmic Cyt C expression (figure 4C, $p < 0.05$) in irradiated cells. In addition, LBP (400 μg/mL) significantly reduced the level of cleaved-caspase 3 (figure 4F, $p<0.05$) and decreased the levels of cleaved-caspase 9 and cleaved -PARP (figure 4E and G, $p<0.01$ and $p<0.05$). These results showed that LBP inhibits the expression of mitochondrial-pathway-associated proteins by irradiated BMNCs.

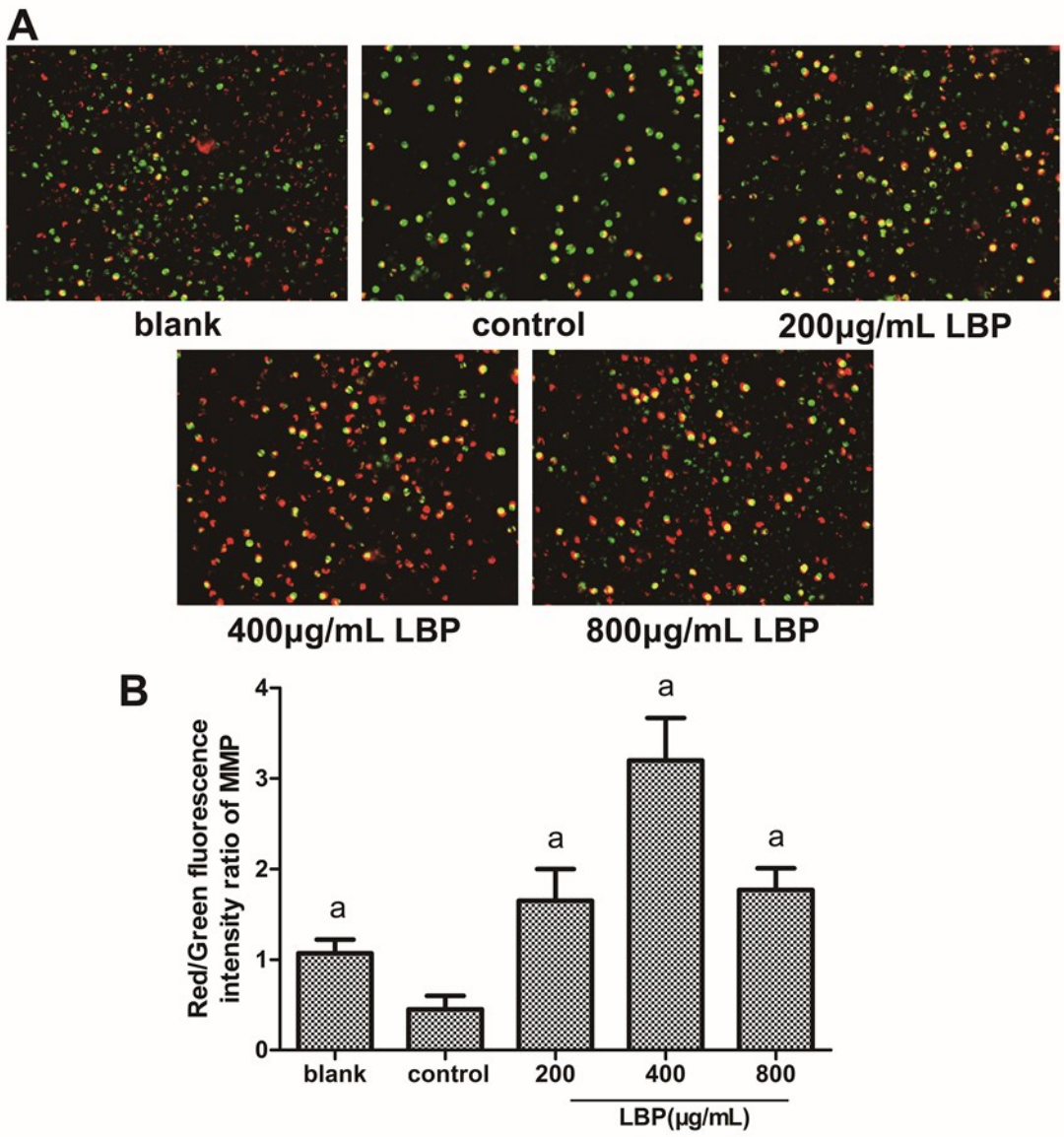

Figure 3. LBP inhibits the decrease in mitochondrial membrane potential of radiation-exposed BMNCs as determined by JC-1 fluorescence assay **(A)**. The data are representative examples of three independent experiments **(B)**. Compared with the control group, [a]$p < 0.01$.

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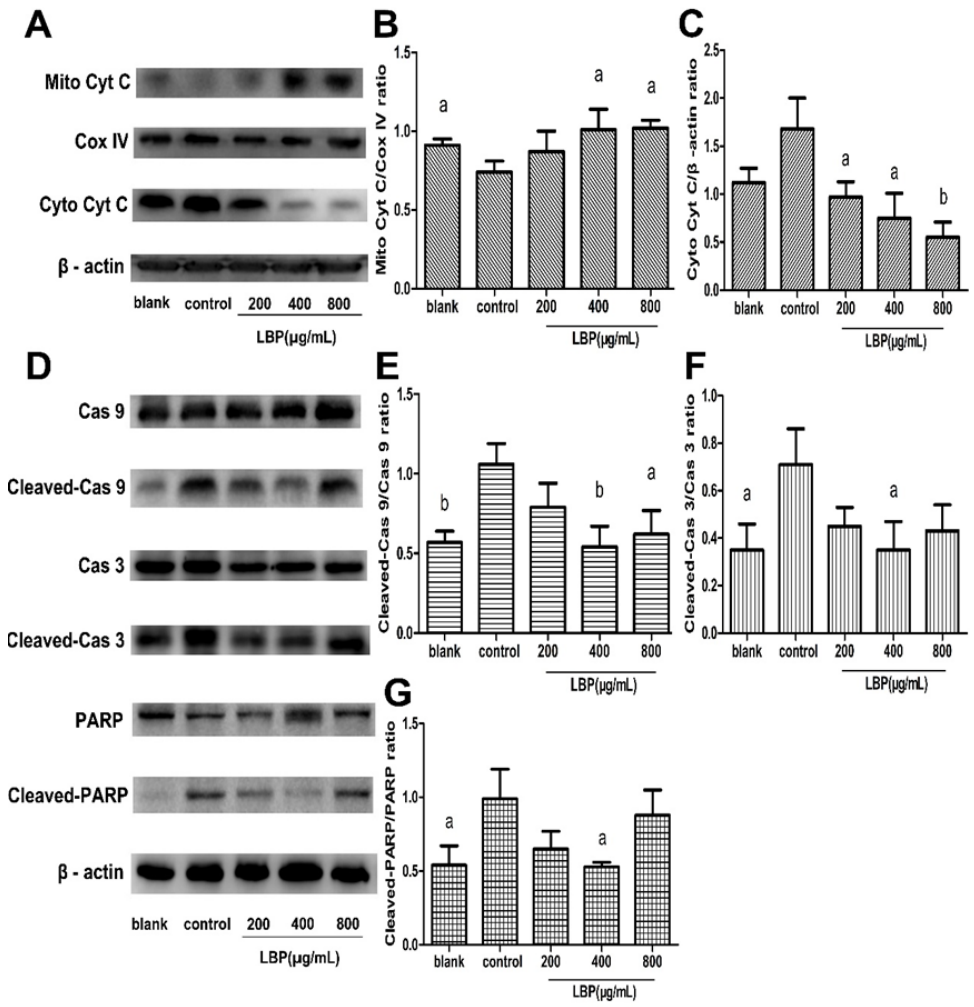

Figure 4. The scanned images show western blots **(A, D)** of Mito Cyt C, Cyto Cyt C, Cleaved-Cas 9, Cleaved-Cas 3 and Cleaved-PARP. The results are expressed as means ± SE of three independent experiments **(B, C, E, F, and G)**. Compared with the control group, [a]p <0. 05, [b]p <0.01.

DISCUSSION

Most synthetic radiation radioprotectants have limited applicability because they are highly toxic at their optimum protective dose. Accordingly, there is increasing interest in natural, nontoxic radioprotectants. For example, UV exposure has been proven to cause the growth of skin fibroblasts to stagnate. Ginkgo biloba extract and other traditional Chinese medicines can reduce damage and promote cell growth [21]. In another study, the radioprotective potential of tomato seed oil in adult male rats exposed to γ-rays was demonstrated [22]. In addition, a recent study reported that melatonin not only has the function of anti-oxidation, but also has the effect of radiation protection. More importantly, melatonin naturally metabolized in the human body, and this is its uniqueness [23].

LBP has been used as a drug in China for centuries and its toxicity is low [24]. Luo *et al.* found that the direct damage of ^{60}Co γ-radiation to spermatogenic cells can be repaired by LBP. In addition, LBP can alleviate the effects of rays on sperm count and sperm motility [1]. Studies in recent years have shown that LBP has a radioprotective effect on the bone marrow system, including a reduction in bone marrow cell apoptosis caused by radiation damage [20]. While the details of the mechanism of action of LBP remain unclear, our results indicate that it is related to the mitochondrial apoptotic pathway. Since LBP is composed of a variety of monosaccharides, whether its actions are attributable to one or more of its active ingredients merits further study.

Radiation-induced apoptosis is often accompanied by specific changes in the plasma membrane, one of the changes is the translocation of phosphatidylserine (PS) from the inside of the plasma membrane to the outside, and then PS is exposed on the outer surface of the cell. Annexin, as a phospholipid binding protein, has a high affinity for PS. So apoptosis can be detected in annexin binding assays [25, 26], as was done in this study. In our study, the apoptosis rate of BMNC cells after LPB treatment was significantly reduced. Mitochondrial membrane potential begins to change in the early stages of apoptosis and can be monitored by JC-1 staining [27].

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The significant increase in the red-green fluorescence ratio of the LBP group demonstrated the efficacy of LBP in preventing the radiation-induced decrease in mitochondrial membrane potential.

Apoptosis, also known as programmed cell death [28, 29], includes an intrinsic pathway mediated by the mitochondria [30, 31]. There are many factors that can cause mitochondrial dysfunction, including certain physical and chemical stimuli, which depolarize the mitochondrial membrane potential. Subsequent events include the entry of CytC into the cytoplasm, where it binds to Apaf-1, recruits caspase 9, activates caspase 3, and cleaves PARP, ultimately leading to cell apoptosis [32]. Our investigation of the molecular mechanism underlying LBP-mediated prevention of apoptosis included determination of the decreased release of CytC from mitochondria to the cytosol and reductions in downstream activated protein (cleaved -caspase 9 *et al.*). The protection against radiation afforded by LBP involves inhibition of the mitochondria-mediated apoptotic pathway. Whether LBP cooperates with multiple signaling pathways related to apoptosis to play a role in radiation protection remains to be determined in further studies.

CONCLUSION

In conclusion, the mechanism of LBP action in radiation-exposed mouse BMNCs cells involves inhibition of the apoptosis and mitochondrial signaling pathway. The results of this study have important significance in the prevention and treatment of radiation damage, and provide a theoretical basis for the development of new radioprotective agents based on LBP and related compounds.

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***Ethical considerations**:* This study was done in compliance with the ethical committee of Chongqing Medical University, Chongqing, China.

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Conflict of Interest: None declared.

Author contribution: (H.H) and (Y.W) contributed equally to this work. (H.H), (Y.W) and (H.P) conceived and designed the study. (H.H), (Y.W), (L.Z), FQH and JL carried out the experiment. HP analyzed the data. (H.H) and (Y.W) drafted and revised the manuscript. All authors read and approved the final manuscript.

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