Preliminary study on the anti-apoptotic mechanism of Astragaloside IV on radiation-induced brain cells

Xin Liu1, Weiwei Chu1, Shuying Shang1, Liang Ma1, Chenxin Jiang1, Yanping Ding2, Jianlin Wang1, Shengxiang Zhang1 and Baoping Shao1

Abstract
With multiple targets and low cytotoxicity, natural medicines can be used as potential neuroprotective agents. The increase in oxidative stress levels and inflammatory responses in the brain caused by radiation affects cognitive function and neuronal structure, and ultimately leads to abnormal changes in neurogenesis, differentiation, and apoptosis. Astragaloside IV (AS-IV), one of the main active constituents of astragalus, is known for its antioxidant, antihypertensive, anti-diabetic, anti-infarction, anti-inflammatory, anti-apoptotic and wound healing, angiogenesis, and other protective effects. In this study, the mechanism of AS-IV against radiation-induced apoptosis of brain cells in vitro and in vivo was explored by radiation modeling, which provided a theoretical basis for the development of anti-radiation Chinese herbal active molecules and brain health products. In order to study the protective mechanism of AS-IV on radiation-induced brain cell apoptosis in mice, the paper constructed a radiation-induced brain cell apoptosis model, using TUNEL staining, flow cytometry, Western blotting to analyze AS-IV resistance mechanism to radiation-induced brain cell apoptosis. The results of TUNEL staining and flow cytometry showed that the apoptosis rate of radiation group was significantly increased. The results of Western blotting indicated that the expression levels of p-JNK, p-p38, p53, Caspase-9 and Caspase-3 protein, and the ratio of Bax to Bcl-2 in radiation group were significantly increased. There was no significant difference in the expression levels of JNK and p38. After AS-IV treatment, the apoptosis was reduced and the expression of apoptosis related proteins was changed. These data suggested that AS-IV can effectively reduce radiation-induced apoptosis of brain cells, and its mechanism may be related to the phosphorylation regulation of JNK-p38.

Keywords
apoptosis, Astragaloside IV, brain, JNK-p38, radiation

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Introduction
With the development of society, people are becoming more and more exposed to radiation due to their treatment, diagnosis, occupation, and accidents. Therefore, the study of the effects of radiation on health has become a very important research field. Radiation causes a variety of damage to human bodies, including immune function decline, cognitive dysfunction, malignant tumors, hematopoietic dysfunction (such as anemia, leukemia, and blood abnormalities), skin ulcers, organ fibrosis, and lens opacity. In addition, radiation also causes multiple damages to living cells, including the loss of genetic information, mutation,
increase genomic instability, and apoptosis. However, the anti-radiation agents currently used in clinical practice have relatively severe toxin and side effects, which limits their application in the clinical treatment of radioactive diseases. Therefore, it has become very urgent to develop a drug that can prevent radiation-induced brain damage with fewer side effects.

Natural drugs are extensively studied as potential neuroprotective agents because of their multi-target and low toxicity characteristics. Saponins, among them, are widely used due to their extensive biological activities, including anti-inflammatory, antibacterial, anti-oxidant, anti-tumor, and neuroprotective effects. Astragaloside IV (AS-IV, 3-O-b-D-xylopyranosyl-6-Ob-D-glucopyranosylcycloastragenol, Figure 1), a cycloalta triterpenoid saponins, is one of the main active ingredients of Astragalus. Known for its protective effects, AS-IV is resistant to oxidation, antihypertensive, antidiabetic, anti-infarction, anti-inflammatory, anti-apoptosis, promotes wound healing and angiogenesis. A large number of literatures show that the anti-apoptotic effect of AS-IV contributes to the improvement of various central nervous system diseases. For example, AS-IV can protect primary cerebral cortex neurons exposed by oxygen and glucose deprivation by regulating the PKA/CREB signal pathway and retaining mitochondrial function. After treatment with AS-IV, the mitochondria and cell damage induced by oxygen and glucose deprivation is reversed, AS -IV significantly enhances the phosphorylation of PKA and cAMP-response element binding protein (CREB) and prevents mitochondrial dysfunction induced by oxygen and glucose deprivation, thereby protecting neurons exposed to oxygen and glucose deprivation from damage and death. AS-IV significantly improved MPTP-induced decrease in primary astrocyte viability, increased apoptosis rate, up-regulation of p-JNK, Bax/Bcl-2 ratio and Caspase-3 activity. AS-IV inhibits Caspase-3 by inhibiting pro-apoptotic p53-mediated Bax activation and anti-apoptotic Bcl-2 activation, thereby improving early brain damage in experimental subarachnoid hemorrhage. Besides, AS-IV can improve the hypoxia-induced damage of PC12 cells by reducing the expression of miR-124. And AS-IV can inhibit the H2O2-induced decrease in the mitochondrial membrane potential of retinal ganglion cells, reduce the release of Cyt c, inhibit the expression of Bax and Caspase-3, and increase the expression of Bcl-2. However, there are few reports on the protective mechanism of AS-IV on radiation-induced neuronal apoptosis. Therefore, this study used radiation-induced Kunming mice and PC12 cells as experimental subjects, and used AS-IV intervention, TUNEL staining, flow cytometry and western blotting to explore the mechanism of AS-IV against radiation-induced apoptosis of brain cells, which provided basic materials for innovative development of anti-radiation active molecules of traditional Chinese medicine and brain health products.

**Materials and methods**

**Animals**

One hundred 30-day-old male Kunming mice (20–25 g) were purchased from the Medical and Laboratory Animal Center of Lanzhou University, and randomly divided into five groups, with 20 in each: blank control group, solvent group (DMSO), DMSO (Solarbio, Beijing, China) + radiation group (DMSO + R), low concentration AS-IV (Dalian Meilun Biotechnology Co. LTD, Dalian, China, ≥98%, BR, CAS NO. MB1955) + radiation group (AS-IV-L + R), high concentration AS-IV + radiation group (AS-IV-H + R). The test was administered by intraperitoneal injection once a day. The control group was given normal saline, both the DMSO group and the DMSO + R group were given DMSO with the final concentration less than 0.01%. The doses of AS-IV-L + R and AS-IV-H + R groups were 20 mg/kg and 40 mg/
kg, respectively. After 1 month of administration, $^{60}$Co radiation (Lanzhou Weite radiation Co., LTD, Lanzhou, China) was used in vivo, and the cumulative radiation dose was 8 Gy\textsuperscript{13-15}.

**Cell culture**

PC12 cells were derived from Lanzhou University School of Basic Medicine and cultured in a humidified incubator containing 5% CO\textsubscript{2} in high glucose medium (Cell Max, Hyclone, Logan City, UT, USA). The high glucose medium contained 5% fetal bovine serum (Gibco, Grand Island, NY, USA), 10% horse serum (Gibco), and 100 U/mL of penicillin with 100 mg/mL of streptomycin (Hyclone). PC12 cells in logarithmic growth phase were collected and divided into five groups: control group (Control), solvent group (DMSO), DMSO + Radiation group (DMSO + R), low concentration AS-IV + radiation group (AS-IV-L + R), high concentration AS-IV + radiation group (AS-IV-H + R). No treatment was performed in the Control group, DMSO was administered to both the DMSO group and the DMSO + R group, with the final concentration less than 0.01%. The doses of AS-IV-L + R group and AS-IV-H + R group were 25 μg/mL and 50 μg/mL\textsuperscript{16-19} respectively. After attaching to 70%-80% of the wall, the cells were irradiated at a vertical distance of 15 cm from the UVA light as radiation source (the cell radiation dose was about 6.5 J/cm\textsuperscript{2}), the irradiation time was 45 min, then treated with the drugs\textsuperscript{13,20-22}.

**TUNEL staining**

The tissue sections were dehydrated gradiently, and after adding the proteinase K diluted with PBS (Solarbio, Beijing, China) dropwise to the sections, the tissues were covered and digested at 37°C for 10 min. Wash PBS three times for 5 min each time. The sections covered with tissue, onto which the labeling buffer added with TdT and DIG-d-UTP and mixed were dropped, were placed in a wet box, labeled and set at 37°C for 2h. Wash PBS three times for 5 min each time. Adding blocking solution and setting at room temperature for 30 min, the sections were washed off but it not washed. Add digoxin antibody diluted with antibody dilution dropwise to the sections until the tissues were covered, so they can be left overnight at 4°C. Wash PBS three times for 5 min each time. Add SABC diluted with antibody dilution dropwise to the sections until the tissues were covered so they can be and incubated at room temperature for 2h in the dark. Wash PBS three times for 5 min each time. Add DAPI staining solution dropwise to the sections for counterstaining and set at room temperature for 10 min. Wash PBS three times for 5 min each time. The plate was mounted with an anti-fluorescence quenching tablet and observed under a fluorescence microscope (Olympus BX53, Tokyo, Japan).

**Flow cytometry**

PC12 cells were seeded at $2 \times 10^5$ cells/well in a 6-well plate. After adhered, the cells were administrated in groups and irradiated according to the above method. After the irradiation, the culture was continued for 24 h at 37°C in an incubator with 5% CO\textsubscript{2}. Each group of cells was collected in a centrifuge tube, centrifuged at 3000 rpm (960 g) for 5 min, washed twice with PBS, and the supernatant of which was removed. Dealt with gentle actions to avoid damage, the cells, were then resuspended in PBS, counted about $1 \times 10^5$, stained by Annexin V-FITC/PI apoptosis detection kit, incubated at room temperature for 10 min in the dark, and then immediately tested on the flow cytometer (BD Biosciences, New York, USA).

**Western blotting**

About 100 mg of brain tissue was placed in a homogenizer, to which a cell lysate containing phenylmethylsulfonyl fluoride (PMSF) (generally 990 μL RIPA plus 10 μL PMSF) was added to the homogenizer, and the tissue samples were placed on ice for rapid homogenization. After ultrasonic crushing, centrifugation was performed at 12,000 rpm for 15 min. Supernatant was extracted and the sample was mixed with 5× protein loading buffer at a ratio of 4:1. Protein samples were separated by SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Boston, MA, USA). The PVDF membranes were incubated at 4°C overnight with the antibodies. After washing and incubating with an HRP-conjugated secondary antibody (Bioss, Beijing, China), the membrane was visualized on hypersensitive chemical fluorescence luminometer by using an ECL reagent.
**Statistical analysis**

Protein data were analyzed by EvolutionCapt for gray value of the bands. GraphPad Prism 5.0 (GraphPad software, San Diego, CA, USA) was used for mapping, using one-way analysis of variance. All data were subjected to standard error and significance analysis. Data were analyzed using SPSS 20.0 statistical software (SPSS Inc., Chicago, IL, USA). Measurement data were expressed by mean ± standard deviation (\( \bar{x} \pm s \)), analyzed by paired t-test. Counting data was expressed as percentage (%), analyzed by paired \( \chi^2 \)-test. \( P < 0.05 \) indicated a statistically significant difference. The data are the means of three independent experiments.

**Results**

**AS-IV attenuated radiation-induced apoptosis of brain cells of mice**

The results of TUNEL staining are shown in Figure 2(a). There were almost no TUNEL-positive cells in the Control group. In the DMSO + R group, a large number of TUNEL-positive cells appeared, while in the AS-IV-H + R group, TUNEL-positive cells were decreased. As shown in Figure 2(b), by counting the apoptotic rate, the apoptosis rate in the DMSO + R group was significantly higher than that in the Control group (\( P < 0.001 \)). Compared with the DMSO + R group, the AS-IV-H + R group (\( P < 0.05 \)) showed a significant decrease of the mortality rate.

**AS-IV attenuated radiation-induced apoptosis of PC12 cells**

To investigate the effect of AS-IV on apoptosis of PC12 cells induced by radiation, the handle cells were subjected to flow cytometry for apoptosis detection of Annexin V-FITC/PI cells. As shown in Figure 3(c), showed that compared with the Control group, the DMSO + R group (\( P < 0.001 \)) presented a significantly higher apoptosis rate. As shown in Figure 3(d), compared with the DMSO + R group, the AS-IV-L + R group (\( P < 0.01 \)) had a decrease apoptosis rate.

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**Figure 2.** TUNEL staining showed the effect of AS-IV on apoptosis of brain cells in mice induced by radiation. (a) TUNEL staining maps of mice brain sections (scale 50 \( \mu \)m). (b) Statistical results of apoptosis rate of mice brain cells. \( n = 3, ***,P < 0.001, *,P < 0.05. \)
Effect of AS-IV on expression of apoptosis-related proteins in brain cells of mice induced by radiation

As shown in Figure 4(a), the specific conditions of AS-IV for apoptosis-induced protein expression in brain cells of mice are as follows. Compared with the Control group, expression of phosphorylated JNK and p38 in the DMSO + R group showed a significant upward trend (Figure 4(c), $P < 0.05$, Figure 4(e), $P < 0.01$). Similarly, expression of p53, Caspase-9, Caspase-3, and the ratio of Bax and Bcl-2 both showed a significant upward trend (Figure 4(f), $P < 0.05$, Figure 4(h), $P < 0.001$, Figure 4(i), $P < 0.001$, Figure 4(g), $P < 0.001$). However, there was no significant difference in protein expression levels between JNK and p38 (Figure 4(b), $P > 0.05$, Figure 4(d), $P > 0.05$). Compared with the DMSO + R group, expression of phosphorylated JNK and p38 in the AS-IV-H + R group showed a significant downward trend (Figure 4(c), $P < 0.05$, Figure 4(e), $P < 0.05$). Expression of p53, Caspase-9, Caspase-3, and the ratio of Bax and Bcl-2 showed a significant downward trend (Figure 4(f), $P < 0.05$, Figure 4(h), $P < 0.001$, Figure 4(i), $P < 0.001$, Figure 4(g), $P < 0.01$). There was no significant difference in protein expression levels of JNK and p38 (Figure 4(b), $P > 0.05$, Figure 4(d), $P > 0.05$).

Effect of AS-IV on expression of apoptosis-related proteins in PC12 cells induced by radiation

The results of the PC12 cells were shown in Figure 5(a). The specific conditions of AS-IV for apoptosis-induced protein expression in PC12 cells are as follows. Compared with the Control group, expression of phosphorylated JNK and p38 showed a significant upward trend in the DMSO + R group (Figure 5(c), $P < 0.001$, Figure 5(e), $P < 0.001$). Similarly, expression of p53, Caspase-9 and Caspase-3, as well as the ratio of Bax and Bcl-2 proteins both showed a significant upward trend (Figure 5(f), $P < 0.001$, Figure 5(h), $P < 0.001$, Figure 5(i), $P < 0.001$, Figure 5(g), $P < 0.01$). However, there was no significant difference in protein expression levels between JNK and p38 (Figure 5(b), $P > 0.05$, Figure 5(d), $P > 0.05$). Compared with the DMSO + R group, expression of
phosphorylated JNK and p38 in the AS-IV-L + R group showed a significant downward trend (Figure 5(c), $P < 0.01$, Figure 5(e), $P < 0.01$). Expression of p53, Caspase-9 and Caspase-3, and the ratio of Bax and Bcl-2 showed a significant downward trend (Figure 5(f), $P < 0.001$, Figure 5(h), $P < 0.001$, Figure 5(i), $P < 0.01$, Figure 5(g), $P < 0.01$). There was no significant difference in
protein expression levels of JNK and p38 (Figure 5(b), $P > 0.05$, Figure 5(d), $P > 0.05$).

**Discussion**

With the rapid increase of the global nuclear power plant construction, the use of radiation therapy in clinical medicine and diagnostic radiology equipment, food sterilization, agricultural research breeding and many other fields, the improper application of which can lead to tissue or organ damage. Therefore, the study of the effect of radiation exposure on our health has become a very important area of research. The central nervous...
Astragalus, one of the most commonly used Chinese herbs, is derived from the roots of astragalus membranaceus. According to Chinese herbs, astragalus can be used as an immune enhancer, liver protection agent, antiperspirant, diuretic and tonic. AS-IV, a kind of cyclopentane triterpenoid saponin, is one of the main active constituents of Astragalus. It is known for its antioxidant, anti-inflammatory, anti-apoptotic, wound healing, vascular regeneration, and other protective effects.

However, few studies have been reported on the anti-radiation effects of AS-IV on brain cells. In this study, we used AS-IV to intervene in mice and irradiate. Through constructing a model of brain cell apoptosis induced by radiation in vivo and in vitro, the AS-IV resistance mechanism of apoptosis induced by radiation was explored.

The mice were intraperitoneally administered daily, after 30 days, 8 Gy 60Co gamma rays were subjected to uniform radiation whole body at one time. It has been reported that radiation can directly damage neuronal progenitor cells, reduce their proliferative capacity and induce apoptosis. Stem cells and neuronal precursor cells that are proliferating are very sensitive to radiation therapy. Ionizing radiation can induce apoptosis, and cause apoptosis as low as 0.25 Gy. TUNEL immunofluorescence staining showed that compared with that of the Control group, the apoptosis rate was significantly increased in the DMSO + R group, indicating that the radiation induced apoptosis of brain cells of mice. Compared with the DMSO + R group, the apoptosis rate of the AS-IV pretreatment re-radiation group at a concentration of 40 mg/kg was significantly decreased, suggesting that AS-IV at a concentration of 40 mg/kg can effectively inhibit radiation-induced brain cell apoptosis. This is consistent with the result that AS-IV can effectively inhibit the apoptosis of hematopoietic cells induced by radiation.

The clonal line, PC12 originally derived from a solid rat adrenal medulla tumor, has been widely used as a dopaminergic neuronal model for in vitro studies of neuronal cell differentiation. When exposed to nerve growth factor (NGF), PC12 cells stop dividing, neural network began and cells became electrically excited to obtain the characteristics of adrenergic neuron phenotype. Therefore, they are similar to mature sympathetic neurons. PC12 cells serve as a principal dopaminergic model in molecular neuroscience for investigating NGF mechanisms of action under normal or after various insults. In addition, the ability to grow PC12 cells in continuous culture with a well-defined secretory cell phenotype has been advantageous for studying secretory pathway mechanisms. In addition to revealing the differentiation into neuronal phenotypes, PC12 cells are also excellent in vitro tools for studying some aspects of various neurological diseases (e.g. glutamate excitotoxicity, Parkinson’s disease, Alzheimer’s disease, and epilepsy). And effect of oxidative stress-related result on neuronal cell survival. Studies using PC12 cells have also solved some problems, such as the impact of serum starvation, NGF deprivation and drug cytotoxicity. So the test in vitro was performed on the neuron-like PC12 cells. After attaching to 70%–80% of the wall, and AS-IV was added, the cells were then subjected to UVA radiation at a dose of 6.5 J/cm² for 45 min. In the early stage of apoptosis, phosphatidylserine is evanescent from the inside of the cell membrane and used as a marker. Flow cytometry combined with FITC-Annexin V/PI fluorescence staining was used to detect radiation-induced PC12 cells. The results showed that compared with that in the Control group, the apoptosis rate was significantly increased in the DMSO + R group, indicating that
radiation induced PC12 cells apoptosis, and the radiation model was successfully established in vitro. Compared with that in the DMSO + R group, the apoptotic rate of the AS-IV pretreatment re-radiation group at 25 μg/mL was significantly decreased, suggesting that AS-IV at concentration of 25 μg/mL can effectively inhibit radiation-induced brain cell apoptosis. This is consistent with the results in vivo. In vitro and in vivo radiation models showed that AS-IV can effectively inhibit radiation-induced brain cell apoptosis. Subsequently, we examined the expression of proteins on the apoptosis-related signaling pathway and found that AS-IV at concentration of 40 mg/kg or 25 μg/mL can effectively inhibit the phosphorylation of JNK and p38, and the content of p53, Caspase-9 and Caspase-3 and Bax/Bcl-2 in brain tissue, and PC12 cells induced by radiation.

MAPK is present in many cells and belongs to the serine/threonine kinase. Many downstream targets of MAPK signaling are involved in neuron development, cell differentiation, cell migration, cancer, cardiovascular dysfunction and inflammation through its role in promoting apoptosis, cell vitality and regulating the functions of various cytokines. MAPK is capable of responding to specific physiological responses caused by a variety of extracellular signals or stimuli, such as radiation, ischemia/reperfusion and inflammation. In mammalian cells, the MAPK family mainly includes three regulatory pathways, namely ERK pathway, JNK pathway and p38 pathway. Studies have found that lipopolysaccharide can induce apoptosis of primary cultured hippocampal neurons through JNK and p38, and the apoptosis is significantly inhibited by JNK inhibitor SP600125 and p38 inhibitor SB202190. Activated by upstream kinase, p38 plays a signal transduction role by acting on a specific substrate. In this experiment, we first observed the effects of radiation on the phosphorylation levels of JNK and p38 in brain tissue and PC12 cells. The results showed that radiation induced the increase of phosphorylated JNK and p38, indicating that radiation can activate JNK signaling pathway. This result is consistent with the expression of JNK and p38 in UV-induced mouse skin. It has also been found that phosphorylation of JNK and p38 is activated in hematopoietic stem cells after irradiation. The phosphorylation levels of JNK and p38 caused by irradiation were effectively inhibited after using AS-IV at concentration of 40 mg/kg or 25 μg/mL in vivo and vitro. Thus, it was concluded that JNK and p38 signaling pathways are involved in the regulation of AS-IV antagonistic of radiation-induced apoptosis in brain tissue and PC12 cells. Study has shown that AS-IV improves apoptosis induced by Aβ25-35-induced endoplasmic reticulum stress by inhibiting p38 signaling pathway in PC12 cells. AS-IV also protects LPS-induced endometritis by inhibiting the activation of p38 and JNK signaling pathways in mouse.

JNK and p38 can directly activate pro-apoptotic Bcl-2 protein to enhance mitochondrial apoptosis pathway, but the phosphorylation of p53 by JNK and p38 is the most important factor in ultraviolet-mediated apoptosis, which delays the degradation of p53 by proteasome and increases the half-life of p53. When exposed to radiation damage, p53 is stabilized and activated by phosphorylation at the Ser15 and Ser20 sites to regulate cell cycle checkpoints and DNA repair. Because p53 can transcribe both pro-survival and pro-apoptotic genes, p53 can initially protect cells from DNA damage after irradiation. If the injury is not repaired, p53 will promote apoptosis, thereby activating the JNK and p38 signaling pathways for a long time, and finally activating the apoptosis pathway. In addition, studies had shown that low doses of radiation induced up-regulation of p53 in embryonic rat brain and induced apoptosis through endogenous pathways. Hematopoietic stem cells (HSCs) of p53-deficient mice were less sensitive to radiation than that of wild-type mice, and p53 inhibitors protected from radiation-induced lethality by inhibiting p53-dependent apoptosis. The Bcl-2 gene is one of the most important anti-apoptotic genes that inhibits apoptosis by regulating the function of the mitochondrial membrane. Bax, another member of the Bel-2 family, has broad amino acid homology with Bcl-2 but functions differently. Bax has an inhibitory effect on apoptosis. Previous reports have shown that the ratio of Bax to Bcl-2 determines, at least to some extent, the sensitivity of cells to death signals. Radiation activates p53, which up-regulates Bax expression, and Bax transfers to the mitochondrial outer membrane, forming a pore across the mitochondrial outer membrane, resulting in a decrease in membrane potential and an outflow of Cyt c and AIF. The change of mitochondrial
membrane permeability leads to the release of Cyt C from mitochondria to cytoplasm and the activation of Caspase-9, which eventually leads to the activation of apoptotic enzyme Caspase-3. Activated Caspase-3 reacts with Caspase-9 pro-enzyme to form a positive feedback pathway. Caspase-3 is the major hydrolase of the apoptotic process and causes apoptosis by hydrolyzing specific protein substrates. Our results show that radiation can increase the ratio of Bax to Bcl-2 and up-regulate the expression of p53, Caspase-9 and Caspase-3 in mouse brain and PC12 cells, which is consistent with the results of endogenous induction of sperm apoptosis in rats by electromagnetic radiation. However, the treatment of AS-IV at concentration of 40 mg/kg or 25 μg/mL can effectively inhibit the increase of p53 in mouse brain tissue and PC12 cells after radiation. Studies have shown that AS-IV can inhibit the expression of apoptosis-related factors (p53, Bax, Caspase-9 and Caspase-3) in retinal ganglion cells induced by oxygen glucose deprivation. This indicated that AS-IV can effectively inhibit radiation-induced apoptosis of brain cells, thereby further preventing radiation damage. However, the specific anti-apoptotic mechanism of AS-IV remains to be further studied.

Conclusion

In summary, AS-IV can effectively reduce the apoptosis of brain cells induced by radiation. The mechanism of AS-IV on radiation-induced apoptosis of brain cells may be related to the phosphorylated regulation of JNK-p38.

Author contributions

X.L. and B.S. designed the study and wrote the manuscript. X.L., Y.D. and B.S. performed the experiments and data analysis. W.C., S.S., L.M. and C.J. participated in the collection of experimental samples. J.W. and S.Z. revised and endorsed the final draft. All authors approved the final manuscript.

Declaration of conflicting interests

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Ethics approval

This study was carried out in accordance with the regulations of Lanzhou University and the ARRIVE guidelines. All experimental procedures and protocols were approved by the Ethics Committee of Lanzhou University.

Animal welfare

All animal studies were approved by the Lanzhou University Institutional Care and Use of Animals.

ORCID iD

Xin Liu https://orcid.org/0000-0002-2100-4778

Data sharing statement

The data used to support the findings of this study are available from the corresponding author upon request.

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