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Experimental Study on the Anti-tumor and Pro-apoptotic Effects of Paeonol in Human Gliomas

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Abstract

Background: Current studies have demonstrated the anti-cancer effects of paeonol in some tumors; however, its effect on gliomas remains unknown.

Objectives: This study aimed to investigate the anti-tumor effect of paeonol in human glioma tissues and cells including its effect and connection with apoptosis and oxidative stress in gliomas.

Methods: Cell Counting Kit-8 (CCK-8) was used to detect the antiproliferative effect of paeonol in human U251 glioma cells. Transwell and colony-forming assays were employed to assess the effect of paeonol on the ability of invasion and colony formation of U251 cells. Superoxide dismutase (SOD) activity, malondialdehyde (MDA) content, total antioxidant capacity (T-AOC), and catalase activity (CAT) were measured to evaluate the effect of paeonol on oxidative stress in U251 cells. Quantitative real-time polymerase chain reaction (RT-q) PCR and western blot were utilized to detect caspase-3 expression levels. Terminal deoxynucleotidyl transferase dUTP nick end-labeling (TUNEL) staining detected the effect of paeonol on U251 cell apoptosis.

Results: Paeonol decreased cell viability, as well as the proliferation, invasion, and colony formation ability of U251 cells. Paeonol reduced MDA content and increased the activities of SOD, CAT, and T-AOC in U251 cells. Caspase-3 expression was lower in human glioma tissues than in normal tissues of the human brain. Paeonol promoted U251 cell apoptosis as revealed by TUNEL staining results and the significant up-regulation of caspase-3 expression in U251 cells.

Conclusion: These results indicated that paeonol has anti-tumor and pro-apoptotic effects in gliomas via oxidative stress regulation and the caspase-3 pathway. Our study, therefore, provides new ideas for the clinical treatment of gliomas.

Keywords: Antioxidants, Apoptosis, Caspase-3, Glioma, Oxidative stress

1. Background

Gliomas are the commonest malignant and fatal intracranial tumors of the central nervous system in adults and are characterized by their rapid infiltrating growth and dysregulated metastasis (1). They account for over 80% of all malignant brain tumors (2). Gliomas are classified by the World Health Organization (WHO) into four categories: Grades I and II are known as "low-grade gliomas" whilst grades III and IV are usually known as "high-grade gliomas". Grade IV is the most aggressive type of glioma and is called glioblastoma multiforme (GBM) (1). Low-grade gliomas are relatively benign and grow slowly; however, although low-grade gliomas initially grow slowly, they usually turn into GBM over time. In addition, studies have shown that about half of patients with low-grade gliomas undergo malignant transformation and transform into GBM within about 5 years (3). The main treatments for glioma include surgical resection, chemotherapy, and radiotherapy (4). However, because gliomas usually show invasive growth and have characteristics of strong invasiveness, even if the patient completely removes the tumor through clinical surgery, it will inevitably recur at the edges of the surgical incision (5). For instance, the current combination treatment method of glioma with cytoreductive surgical treatment, chemotherapy, and radiotherapy only extends lifespan by an average of months (6). Therefore, gliomas are difficult to completely remove by surgery. Even more frightening is the fact that the therapeutic effects of radiotherapy and chemotherapy in the treatment of gliomas are not very effective (7). Although it is quite heartwarming that emerging immunotherapeutic modes of treatment seem to have made some gratifying progress in the treatment of glioma, they are still in the stage of clinical trials (8). Moreover, the threat of glioma to human health is becoming more serious with the increasing aging population; therefore, early diagnosis and treatment of gliomas have become a vital medical issue with the hunt for new drugs to treat gliomas being very imperative.

Most current experimental studies used the induction of cell death and the inhibition of growth as the main cancer therapeutic approach since anti-apoptosis and unlimited proliferation are two vital characteristics of gliomas (9-13). In addition, numerous recent experimental and clinical research evidence shows that oxidative stress occurs in the development of glioma, and oxidative stress may be

an indispensable contributor to the proliferation and migration of glioma cells (11, 14-17).

Compared to normal brain tissues, the activity of free radical scavenger catalase (CAT) in glioma tissues is increased, but the activity of superoxide dismutase (SOD) is decreased (18). Moreover, it has also been found that targeting oxidative stress with some molecules in glioma cell lines reduces the invasion and proliferation of glioma cells (19). Since the important role of oxidative stress in tumors indicates that it may be a potential target in cancer treatment, some antioxidants that play critical roles in the response to oxidative stress have received a lot of attention in the study of cancer lately (20). Antioxidation systems are classified into enzymatic and non-enzymatic anti-oxidant groups. The non-enzymatic group consists of numerous anti-oxidants that directly act on oxidative agents and are normally derived from dietary sources. The non-enzymatic group includes flavonoids, polyphenols, vitamin C, carotenoids, and vitamin E whilst the enzymatic group is made up of several enzymes, such as CAT and SOD. SOD and manganese (Mn) work together in the mitochondrion whilst Zinc (Zn)/Copper (Cu) works in the cytosol and serves as a catalyst involved in breaking down superoxide-anions into hydrogen peroxide (H_2O_2) and oxygen (21). In the presence of Mn/Iron (Fe) cofactors, CAT serves as a catalyst in converting H_2O_2 to water and oxygen (21).

A recent study revealed that the oxidative stress response which is induced by the stimulation of H_2O_2 inhibits the growth of human U251 glioma cells and induces glioma cell apoptosis through a caspase-3 dependent pathway (22) and is therefore an indication that in gliomas, the process of apoptosis mediated by oxidative stress may play a crucial role.

Paeonol ($C_9H_{10}O_3$,2′-hydroxy-4′-methoxyacetophenone) is the principal active compound in the extract of peony root bark used in traditional Chinese medicine for the improvement of fever, dysmenorrhea, amenorrhea, and blood circulation (23, 24). Paeonol has free-radical-scavenging and anti-oxidative effects and recent studies have shown it has anti-tumor effects in many cancers (25-27).

2. Objectives

This study mainly investigated the anti-cancer and anti-oxidant effects of paeonol in gliomas by exploring the therapeutic potential of paeonol against gliomas, as well as its potential mechanisms of action in the treatment of gliomas.

3. Methods

3.1.Preparation of Paeonol

Institutional Review Board (IRB) approval was obtained (IRB No. 20210907-JGSU) for this study. Paeonol was obtained from YuanYe Biological (AB22173, Shanghai, China). It was dissolved in DMSO (ST038, China Beyotime Biotechnology) and prepared into a 10M paeonol solution and stored at -20°C.

3.2.Cell culture

Human glioma cell line U251 was purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). Culturing of cells was performed in DMEM (11965092, Thermo Fisher Scientific, USA) that contains 10% FBS (12483020, Gibco, Canada) and 1% penicillin/streptomycin (C0224, China Beyotime Institute of Biotechnology) in an incubator at 95% humidity, 37°C, and 5% CO2. Logarithmic-growth-phase cells were used in our experiment.

3.3. Cell counting kit (CCK-8) assay

In order to verify paeonol's cytotoxicity on U251 cells, CCK-8 (C0038, China Beyotime Institute of Biotechnology) was used in accordance with the manufacturer's protocol to determine the cell viability and growth curve of U251 cells using different concentrations of paeonol. Logarithmic growth phase U251 cells were seeded into a 96-well plate at a density of 5×10^3 cells per well and then cultured for 12, 24, 36, 48, and 72 hours. CCK-8 solution (10μL) was added to the wells before incubation for 1 hour at 37°C. Absorbance was then measured at 450 nm wavelength (OD450) in a microplate reader (Biorad; Synergy 2).

3.4. Colony formation assay

A colony formation assay was employed to evaluate the inhibitory effect of paeonol on the growth of U251 cells. U251 cells were seeded in a 3.5-cm culture dish at a density of 200 cells per dish before incubation for 24 hours. The medium was changed to a fresh medium that contains paeonol (0, 50, 100, 200μM) and cultured for 15 days (medium refreshed every 3 days). It was then washed two times with PBS, fixed with 4% paraformaldehyde (P0099, China Beyotime Biotechnology) for 15 minutes, and then washed twice again with PBS. Staining with 1% crystal violet (C0121, China Beyotime Biotechnology) was performed for 5 minutes. Finally, it was washed and air-dried, and then, the cells were observed.

3.5. Transwell invasion and migration assays

Transwell invasion and migration assays were used to evaluate paeonol's effect on the invasiveness of U251 cells. In short, transwell inserts were coated with matrigel which was diluted with serum-free medium. After U251 cells were trypsinized and washed, they were resuspended in the culture DMEM medium containing 20% FBS with the various appropriate concentrations of paeonol (1×10^5/mL). After that, 100 μL of cell suspensions was added to the upper transwell compartment, followed by the

addition of 600 µL of DMEM with 20% FBS into the lower compartment. Cells in the transwell plate were incubated with 5% CO_2 for 24 hours at 37°C. Cells on the lower side of the insert filter were fixed with 10% methanol and stained with 0.1% crystal violet after the cells that remained on the upper side of the filter membrane had been removed with a cotton swab. Images of the cells on the lower side of the insert filter were taken under an inverted microscope (Leica, Germany).

3.6.Western blotting

Western blot was used to detect the expression of caspase-3 in U251 cells in the control group and paeonol treatment groups. U251 cells cultured with different concentrations of paeonol were harvested and lysed for the extraction of proteins with cell lysis buffer (P0013, China Beyotime Biotechnology). Samples of protein were separated using an SDS polyacrylic gel and transferred to a PVDF membrane. The membranes were blocked with 5% skimmed milk powder. Incubation of samples with caspase-3 (1:1000, AF6311, Affinity-USA) and β-actin (1:4000, AF7018, Affinity-USA) primary antibodies was then carried out. They were subsequently incubated with HRP-conjugated secondary antibody (1:5000, S0001, Affinity, USA). After the development of blots with enhanced chemiluminescence solution (China Beyotime Biotechnology), protein band optical density was then analyzed with Tanon Protein Analysis System.

3.7. Immunohistochemistry

Immunohistochemistry was used to evaluate caspase-3 protein expression in human glioma tissues. Tissue slices (t147b, Alena Biotechnology, China) were dewaxed and hydrated in xylene, ethanol, and water, and then washed with PBS. For antigen retrieval, paraffin slides were put in citrate antigen retrieval solution and the cells were washed 3 times with PBS for 3 minutes each before being blocked with 10% goat serum for an hour. Incubation was performed with primary antibodies caspase-3 (1:200, Affinity, USA) overnight at 4°C. After primary antibody incubation, it was washed 3 times with PBS for 3 minutes each. Secondary antibodies were then added to the slides before a room temperature incubation for 1 hour. It was then washed thrice with PBS for 3 minutes. Finally, the nucleus was counterstained with DAB and hematoxylin for 5 minutes.

3.8. QRT- PCR

QRT-PCR was used to detect the expression of caspase-3 in U251 cells in the control and paeonol treatment groups. We used TaKaRa-MiniBEST-Universal-RNA Extraction Kit to extract the total RNA from U251 cells treated with different concentrations of paeonol following the manufacturer's protocol (Takara-Bio, China, Cat.9767). cDNA was synthesized with the use of the Prime-Script-RT-Master-Mix kit in accordance with the manufacturer's (Takara, China, RR036a) protocol. qRT-PCR was then performed using TB-Green™-Premix Ex-Taq™ II (Takara, China, RR820a) by adhering to the manufacturer's instructions. The 2-ΔΔCq method was used to analyze relative gene expression and GAPDH expression served as the internal control. The primers used are provided in Table 1.

Table 1. Primers for QRT-PCR used in this study

Primer Name	Sequence (5'-3')
Caspase-3 (Forward)	TTCAGAGGGGGATCGTTGTAGAAGTC
Caspase-3 (Reverse)	CAAGCTTGTCGGCATACTGTTTCAG
GAPDH (Forward)	CTGGTAAAGTGGATATTGTTGCCAT
GAPDH (Reverse)	TGGAATCATATTGGAACATGTA AACC

QRT- PCR: Quantitative real-time polymerase-chain-reaction

3.9. CAT, SOD, Total Antioxidant Capacity (T-AOC), and Malondialdehyde (MDA) measurement

SOD, CAT, T-AOC, and MDA were measured to test paeonol's effect on the oxidative stress level of U251 cells. In short, after U251 cells that were cultured with different concentrations of paeonol were harvested, measurement of the content/activity of CAT, MDA, SOD, and T-AOC was carried out with an MDA kit (S0131S, China Beyotime Biotechnology), SOD kit (S0101S, China Beyotime Biotechnology), CAT kit (S0051, Beyotime Biotechnology, China), and T-AOC kit (S0116, China Beyotime Biotechnology) following the manufacturer's instructions.

3.10.Cell apoptosis detection by Terminal deoxynucleotidyl transferase dUTP nick end-labeling (TUNEL) staining

TUNEL staining was used to evaluate paeonol's effect on U251 cell apoptosis. U251 cells in the logarithmic growth phase were seeded in a 24-well plate at a density of 1×10^5 cells per well and cultured in fresh medium containing paeonol (0, 50, 100, 200 µM) for 24 hours. The cells were stained with TUNEL assay with the use of a cell death detection kit (Thermo Fischer) in strict accordance with the manufacturer's protocol. The apoptotic index was calculated as the cell number of TUNEL-positive nuclei divided by the total number of cells across the viewed areas and multiplied by 100.

3.11.Statistical analysis

Data were expressed as mean±SD. Assessments of comparisons were made with one-way analysis of variance (ANOVA) and Tukey's post hoc test using SPSS 17.0 (Chicago-IL, USA). $P<0.05$ was deemed statistically significant.

4. Results

4.1. Paeonol inhibits viability, proliferation, the ability of invasion, and colony formation of U251 cells

In order to verify paeonol's cytotoxicity on U251

cells, CCK-8 was employed to determine the cell viability and growth curve of U251 cells at 24, 48, and 72 h using different concentrations of paeonol.

As seen in Figure. 1A, paeonol inhibited the cell viability of U251 cells, and this inhibitory effect gradually increased as the concentration of paeonol increased. For instance, U251 cells had a viability of (48.82±2.32) (%) when the concentration of paeonol was 200μM, but it was only (38.3±1.6) (%) when the concentration of paeonol was 400μM. Subsequently, according to these results, paeonol concentrations of 50μM, 100μM, and 200μM were regarded as low, medium, and high concentrations of paeonol, respectively, for further analysis in this study. Therefore, 0μM Con was referred to as the control group, whilst 50μM LP, 100μM MP, and 200μM HP were referred to as low, medium, and high concentrations of paeonol treatment groups, respectively. To test the effect of paeonol on U251 cells at non-cytotoxic doses, we conducted cell colony formation, as well as transwell invasion and migration experiments. It was revealed that compared to the control group, the colony-forming ability (Figure.1B and 1C) of U251 cells treated with paeonol all decreased. Moreover, 100μM MP and 200μM HP groups showed a significant decrease in colony-forming ability, compared to the control group. It was also revealed that the invasiveness (Figure.1D) of U251 cells treated with paeonol all significantly decreased in comparison with the control group. These results therefore indicate that paeonol inhibits the colony-forming and invasion ability of human U251 cells.

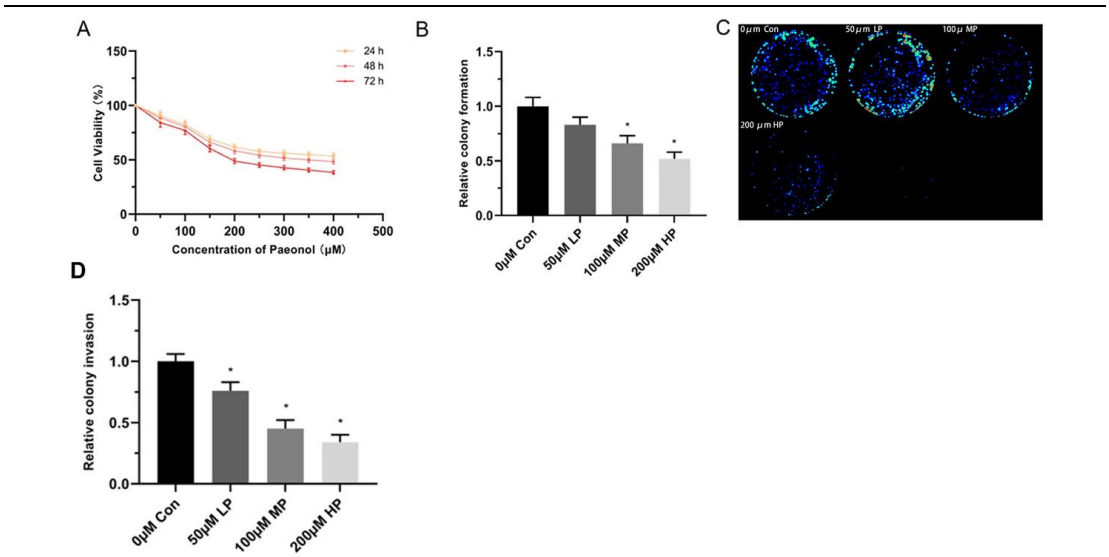

Figure 1. Effects of Paeonol on U251 Cells. (A) Results showing that paeonol inhibits cell viability and proliferation ability of U251 cells in a dose-dependent fashion. (B, C) Results showing that paeonol inhibits the colony-forming ability of U251 cells in a dose-dependent fashion. (D) Results showing that paeonol inhibits the invasion ability of U251 cells in a dose-dependent fashion
Data are expressed as mean±SEM, (n=3), *P＜0.05 versus 0μM Con

4.2. Paeonol reduces oxidative stress levels in U251 cells

For the purpose of testing paeonol's effect on the oxidative stress level of U251 cells at non-cytotoxic doses, we measured SOD, CAT, T-AOC, and MDA. As shown in Figures 2A, 2B, and 2C, compared to the 0μM Con group, the SOD activity, CAT activity, and T-AOC of the 50μM LP, 100μM MP, and 200μM HP groups were significantly improved. Figure 2D also revealed that compared to the 0μM Con group, the MDA content in the 100μM MP and 200μM HP groups were significantly decreased. These results demonstrate paeonol's anti-oxidative effect in U251 cells.

4.3. Paeonol reduces the expression of caspase-3 in gliomas

To study whether paeonol can inhibit the growth of human U251-glioma cells by regulating caspase-3 to induce apoptosis in glioma cells, caspase-3 protein expression was initially evaluated in human glioma tissues using immunohistochemistry technique. As shown in Figures 3A and 3B, caspase-3 expression level in human glioma tissues is decreased, compared to normal human tissues. qRT-PCR and western blot were then used to detect the expression of caspase-3 in U251 cells in the control group and paeonol treatment groups. As shown in our western blot results (Figures 3C and 3D), compared to the 0μM Con group, caspase-3 expression levels in U251 cells treated with paeonol were all significantly higher. Our qRT-PCR results (Figure 3E) revealed that compared to the 0μM Con group, the expression level of caspase-3 in the 100μM MP and 200μM HP groups was significantly higher. Therefore, the inhibitory effect of paeonol on U251 cells could be attributed at least in part to the increase in caspase-3 expression.

4.4. Paeonol promotes U251 cell apoptosis

To verify paeonol's effect on the apoptosis of U251 cells, an apoptosis-TUNEL staining kit was used to detect the rate of apoptosis of U251 cells. As revealed in Figures 4A and 4B, compared to the 0μM Con group, the rate of apoptosis in the 50μM LP, 100μM MP, and 200μM HP groups was significantly increased. Moreover, this effect is enhanced with the increase in paeonol concentration. These observations indicate that paeonol promotes the apoptosis of U251 cells.

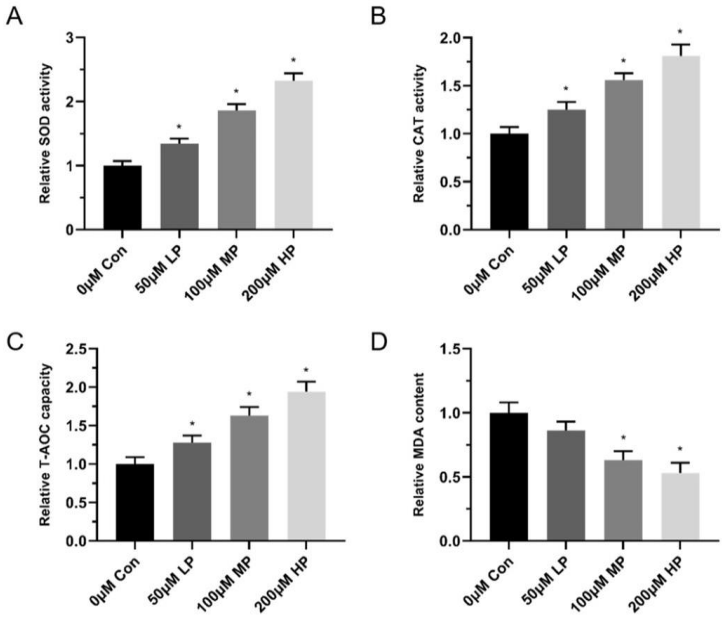

Figure 2. Effects of paeonol on the oxidative stress level of U251 Cells. (A) Paeonol increased SOD activity, (B) increased CAT activity, (C) increased T-AOC activity, and (D) reduced MDA content in U251 cells in a dose-dependent fashion
Data are expressed as mean±SEM, (n=3), *P＜0.05 versus 0μM Con

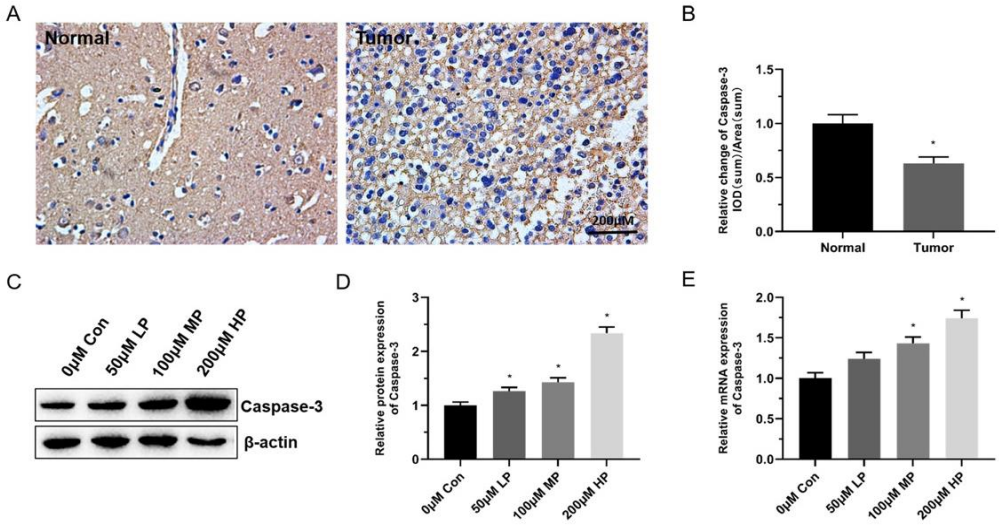

Figure 3. Effect of paeonol on Caspase-3 in gliomas. (A, B) Results showing that the expression level of Caspase-3 in glioma tissues was significantly decreased, compared to normal tissues. (C, D) Western blot results showing that paeonol upregulates the expression level of caspase-3 in U251 cells in a dose-dependent fashion. (E) qRT-PCR results showing that paeonol upregulates the expression level of caspase-3 in U251 cells in a dose-dependent fashion
Data are expressed as mean±SEM, (n=3), *P＜0.05 versus 0μM Con

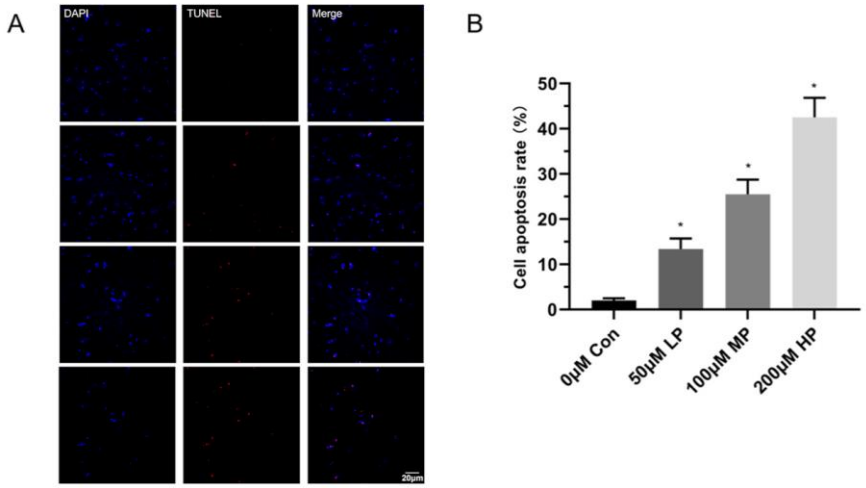

Figure 4. Effect of paeonol on U251 cell apoptosis. (A, B) Results showing that paeonol increases the apoptosis rate of U251 cells in a dose-dependent fashion
Data are expressed as mean±SEM, (n=3), *P＜0.05 versus 0μM Con

5. Discussion

In our present study, it was found that paeonol inhibits the colony-forming and invasion ability of human U251 cells, promotes apoptosis by the up-regulation of caspase-3 expression in U251 cells, and exerts an anti-oxidative effect in U251 cells by reducing MDA content and increasing the activities of SOD, CAT, and T-AOC.

Although gliomas are one of the most common and deadly intracranial tumors, their specific pathogenesis is still unclear. Recent articles have explained that injuries caused by oxidative stress are important factors in the formation and development of cancer, and therefore, some substances with anti-oxidant activity inhibit the invasion and migration of glioma cells by regulating oxidative stress (11, 16, 17, 28, 29). Because paeonol also has antioxidant properties (30), it was hypothesized that paeonol is also capable of inhibiting the growth, invasion, and migration of glioma cells via oxidative stress regulation. In order to verify this hypothesis, the CCK8 method was first used to detect the effect of paeonol below cytotoxic levels on the growth and proliferation of U251 cells. It was revealed in our results that paeonol can significantly reduce the viability and proliferation ability of glioma cells which is a strong indication that paeonol exerts an inhibitory effect on the growth of glioma cells.

In addition, because gliomas have a strong invasion ability, we used a transwell invasion test and colony-forming assay to test paeonol's effect on the invasion ability and viability of glioma cells. Our results also revealed that paeonol can significantly reduce the invasion ability and colony-forming ability of glioma cells which therefore shows that paeonol can inhibit the colony-forming ability and invasion ability of glioma cells. Paeonol's anti-cancer effects demonstrated in our current study have also been confirmed by Zhang L et al. in lung cancer cells (31).

With the recent realization of oxidative stress playing an important role in cancer (28, 32) and more specifically in the development of glioma through numerous experimental and clinical research evidence (22, 33), we tested SOD activity, CAT activity, T-AOC, and MDA content in different groups of glioma cells after our earlier confirmation of the anti-cancer effect of paeonol on gliomas in our study. Our results clearly indicated that paeonol exerts an anti-oxidant effect in gliomas and inhibits oxidative stress evidenced by its ability to significantly increase SOD activity, CAT activity, T-AOC and reduce MDA content in glioma cells, all in a dose-dependent fashion. Our results were in harmony with the findings of other studies that also showed paeonol's anti-oxidant properties (34-37). Our study, therefore, confirms that the anti-oxidant effect of paeonol plays an important role in its anti-cancer effect in gliomas.

It is a well-known fact that apoptosis is very vital in cancer research and has become the main focus of glioma studies as well (38-40). Moreover, studies have shown that oxidative stress inhibits the growth of human U251 glioma cells and induces glioma cell apoptosis via a caspase-3-dependent pathway (22). Therefore, in the present study, we first measured caspase-3 expression in human glioma tissues using immunohistochemistry and found out that caspase-3 expression in human glioma tissues was lower than that in normal tissues. We then used Western blotting and qRT-PCR to detect the effect of paeonol on caspase-3 in glioma cells. Interestingly, it was revealed that paeonol significantly upregulates caspase-3 expression in glioma cells. For more clarity, we further used TUNEL staining technique to detect paeonol's effect on the rate of apoptosis in glioma cells. Our results showed that paeonol increases the apoptotic rate of glioma cells in a dose-dependent fashion. Taken together, the above results indicate

that the apoptosis effect of paeonol in glioma cells may be caused by the caspase-3 pathway. Furthermore, although the design of the present study revealed the anti-tumor effects of paeonol through its pro-apoptotic effect in human gliomas by the upregulation of caspase-3, we believe the quality of data can be improved in future studies by designing investigations revealing more explicit mechanisms of how paeonol stimulates caspase-3 up-regulation.

6. Conclusion

In summary, this experiment proves that paeonol has anti-cancer and pro-apoptotic effects in gliomas, and these effects may be accomplished by oxidative stress regulation and via the caspase-3 pathway. Our study, therefore, provides new ideas for the clinical treatment of gliomas.

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Footnotes

Conflicts of Interest: The authors report no conflicts of interest.

Authors' Contribution: EA and DHL: study design, experimental implementation, and manuscript drafting. QF and XZL: supervision of all experiments and data interpretation. XPZ and CM: experimental implementation and data collection. EA and DHL: data analysis and manuscript revision. All authors read and approved the manuscript.

Ethical Approval: Institutional Review Board (IRB) approval was obtained (IRB No. 20210907-JGSU) for this study.

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