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Clinical effects of *Angelica dahurica* dressing on patients with I-II phase pressure sores

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Objective: Angelica dahurica is a well-known traditional Chinese Medicine (TCM), while little information is available about its effects on pressure sores. We aimed to investigate the clinical effect of *Angelica dahurica* on patients with I-II phase pressure sores, as well as the underlying mechanism. *Methods:* Patients (n = 98) with phase I and phase II pressure sores were enrolled and randomly assigned to control and treated groups. In addition to holistic nursing, patients in the control group received compound clotrimazole cream, while patients in the treated group received continuous 4 weeks of external application of *Angelica dahurica* dressing. Therapeutic effect was recorded, along with the levels of interleukin-8 (IL-8), epidermal growth factor (EGF), transforming growth factor (TGF)-β, and vascular endothelial growth factor (VEGF). Besides, HaCaT cells were cultured with different concentrations of Angelica dahurica, and then cell viability, clone formation numbers, cell cycle, and levels of cyclin D1 and cyclin-dependent kinase (CDK) 2 were determined. *Results:* The total effective rate in the treated group was significantly higher than in the control group. Levels of IL-8, EGF, TGF-β, and VEGF were statistically increased by *Angelica dahurica*. In addition, the cell viability and clone formation numbers were significantly upregulated by Angelica dahurica in a dose-dependent manner. Also, the percentage of cells in G0/G1 phase, and levels of cyclin D1 and CDK2 were significantly elevated.
Conclusion: Our results suggest that *Angelica dahurica* may provide an effective clinical treatment for I-II phase pressure sores.

1. Introduction

Pressure sores are localized damages in the skin and/or underlying tissue resulting from unrelieved pressure over a bony prominence, affecting the physical and psychological health of patients (Cushing and Phillips 2013; Aydin and Mucuk 2015). Pressure sores are also prone to infection, which could spread to the bones and blood stream, making them a big challenge to treat. Although a remarkable effort has been made in recent years to prevent and treat pressure sores, many patients especially the elderly, critically ill, spinal cord-injured are still vulnerable, leading to increase of hospitalization time and medical expenditure, and tremendous decrease of quality-of-life (Karadağ 2003; Cushing and Phillips 2013). Moreover, the incidence rate and prevalence rates of pressure sores have been reported to be rapidly increased due to the growth of aging population and chronic diseases (Davis and Caseby 2001; Whittington and Briones 2004; Scott et al. 2006; Tannen et al. 2006; Jiang et al. 2014). Therefore, there is an urgent need to develop new effective intervention and appropriate treatment to prevent and treat pressure sores.

Recently, Traditional Chinese Medicine (TCM) has been paid a great attention and widely used in treating pressure sores (Zhang et al. 2013). Angelica dahurica is a well-known TCM, which has long been used for many years to treat headache, toothache, cancers, abscess, furunculosis, and acne (Kim et al. 2007; Wang et al. 2011; Li et al. 2014). Angelica dahurica displays a variety of bioactivities such as antimicrobial (Kwon et al. 1997), anti-asthmatic (Lee et al. 2011b), anti-inflammatory (Lee et al. 2011a), antioxidant (Piao et al. 2004), anti-tumor (Luo et al., 2011), and anti-proliferation (Kim et al. 2007) effects. However, little information is available with respect to the clinical effects of Angelica dahurica on pressure sores, as well as the underlying mechanism.

Therefore, the present study was aimed to investigate the clinical effects of Angelica dahurica on pressure sores and tried to elucidate the underlying mechanism. We compared the therapeutic effects of Angelica dahurica with compound clotrimazole cream dressing on the basis of holistic nursing. The underlying therapeutic mechanism was explored *in vitro* and *in vivo*.

2. Investigations and results

2.1. Comparison of clinical data

No significant differences were observed among the gender, age, and average duration between the two groups. The results were comparable. To investigate the efficacy of Angelica dahurica dressing on pressure sores, patients in the treated group received continuous 4 weeks of treatment with Angelica dahurica dressing, and while patients in the control group were administrated with compound clotrimazole cream. Then we compared the therapeutic effects between the two groups. There were 34 healing cases, 10 markedly effective cases, 5 effective cases, and none ineffective case in the treated group, respectively. There were 21 healing cases, 11 markedly effective cases, 10 effective cases, and 7 ineffective cases in the control group, respectively. The total effective rate was 91% and 65% in the treated and control group, respectively, and a significant difference was found between the two groups ($P < 0.05$).

2.2. Effects of Angelica dahurica on expression of growth factors and cytokines

We then examined the expression of growth factors and cytokines (IL-8, EGF, TGF-β, and VEGF) between the two groups. The tissues were obtained from both the two groups, and the expression of IL-8,

EGF, TGF-β, and VEGF were analyzed by ELISA. As indicated in Fig. 1 A-D, the results showed that compared to the control group, the expression levels of IL-8, EGF, TGF-β, and VEGF were all significantly increased in the treated group ($P < 0.05$ or $P < 0.01$). The results demonstrated that Angelica dahurica promoted regeneration by upregulating the expression of growth factors and cytokines.

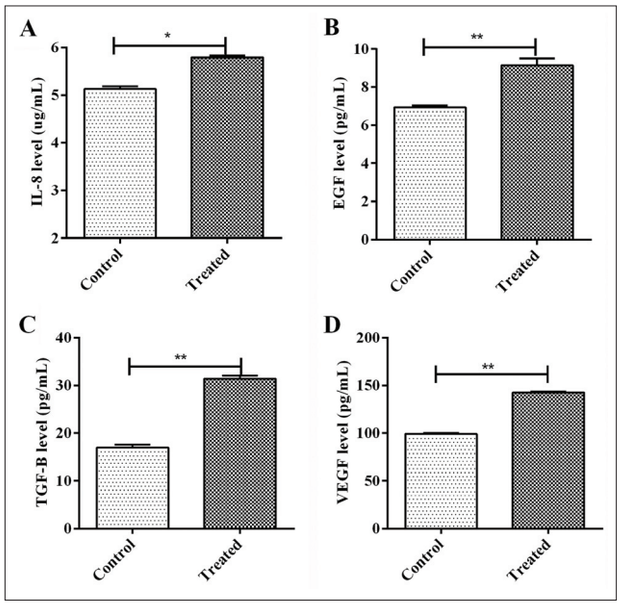

Fig. 1: Effects of Angelica dahurica on expression of growth factors and cytokines. The expression of IL-8 (1 A), EGF (1 B), TGF-β (1 C), and VEGF (1 D) was determined between the control group and treated group by ELISA. The results showed that the expression levels of IL-8, EGF, TGF-β, and VEGF were all significantly increased by Angelica dahurica compared to the control group. ELISA, enzyme-linked immuno sorbent assay; IL, interleukin; EGF, epidermal growth factor; TGF, transforming growth factor; $^{*}P < 0.05$ or $^{**}P < 0.01$ compared to the control group

2.3. Effects of Angelica dahurica on cell growth

Next, we performed *in vitro* experiments to explore the effects of Angelica dahurica on cell growth by analyzing cell viability and colony formation ability. Human HaCaT cells were cultured and treated with different concentrations of Angelica dahurica (1 mg/ml, 2 mg/ml, and 4 mg/ml). Thereafter, the cell growth was assessed by MTT and colony formation assay. The results showed that 1 mg/ml Angelica dahurica had no significant influence on the cell viability and colony formation numbers. However, the cell viability and colony formation numbers were statistically upregulated by 2 mg/ml and 4 mg/ml Angelica dahurica ($P < 0.05$ or $P < 0.01$) (Fig. 2 A and B), and the effect was shown in a dose-dependent manner. The results indicated that Angelica dahurica promoted cell growth.

2.4. Effect of ligustrazine on cell cycle

Based on the above results, we furthermore studied the promotive cell growth by determining the effect of Angelica dahurica on cell cycle. After HaCaT cells had been treated with different concentrations of Angelica dahurica (1 mg/ml, 2 mg/ml, and 4 mg/ml), the percentage of cells in G0/G1, S, and G2/M phases were analyzed. As shown in Fig. 3 A, we observed that 2 mg/ml and 4 mg/ml Angelica dahurica could significantly increase the percentage of cells in G0/G1 phase ($P < 0.05$ or $P < 0.01$). The levels of cell cycle-related protein cyclin D1 and CDK2 were determined by qRT-PCR and Western blotting. The results revealed that both the mRNA levels of Cyclin D1 and CDK2 were significantly elevated by Angelica dahurica (1 mg/ml, 2 mg/ml, and 4 mg/ml) ($P < 0.05$ or $P < 0.01$) (Fig. 3 B). Similarly, both the protein levels of cyclin D1 and CDK2 were statistically increased by Angelica dahurica (Fig. 3 C). The results suggested that Angelica dahurica promoted cell growth by acceleration of the cell cycle process.

3. Discussion

In the present study, we explored the clinical effects of Angelica dahurica dressing on patients with I-II phase pressure sores and investigated the underlying mechanism. The main findings of the study suggested that Angelica dahurica provided a better effective clinical treatment than the patients who received compound

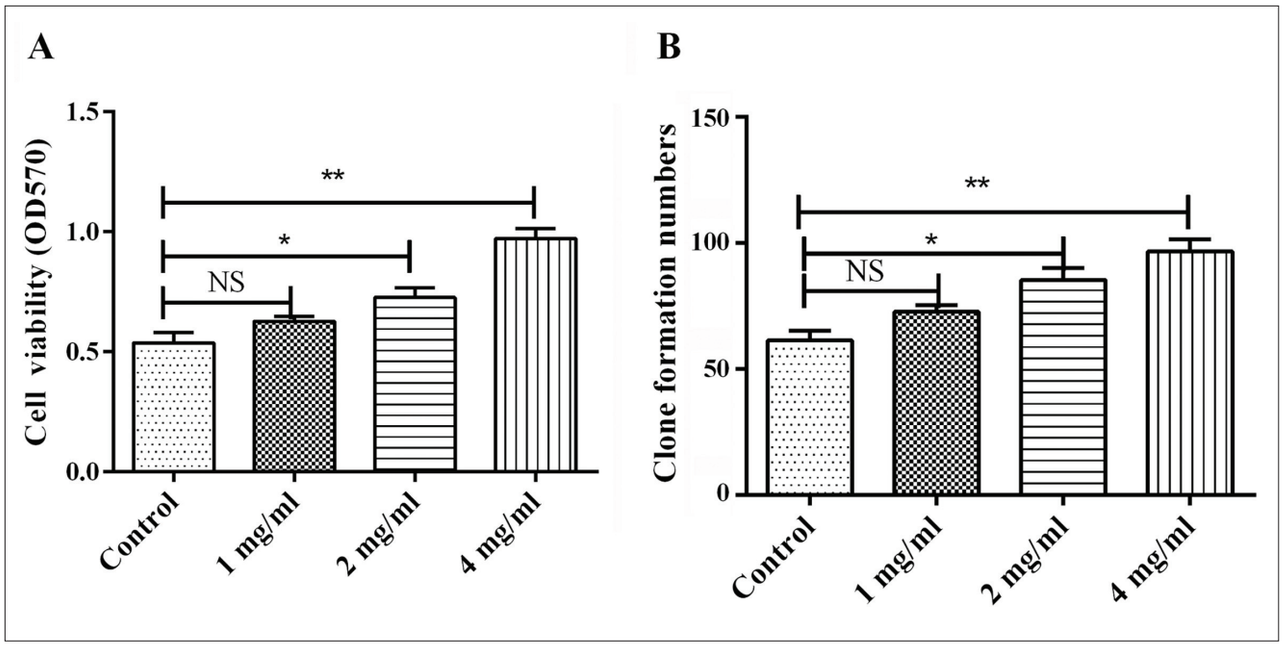

Fig. 2: Effects of Angelica dahurica on cell growth. The cell growth was assessed by MTT (2 A) and colony formation assay (2 B) after administration with different concentrations of Angelica dahurica (1 mg/ml, 2 mg/ml, and 4 mg/ml). The results demonstrated that the cell viability and colony formation numbers were statistically upregulated by 2 mg/ml and 4 mg/ml Angelica dahurica, and the effect was in a dose-dependent manner. MTT, 3-(4, 5-dimethylthiazol-2-yl)-2, 5- diphenyltetrazolium bromide; NS, no significance; OD, optical density; $^{*}P < 0.05$ or $^{**}P < 0.01$ compared to the control group

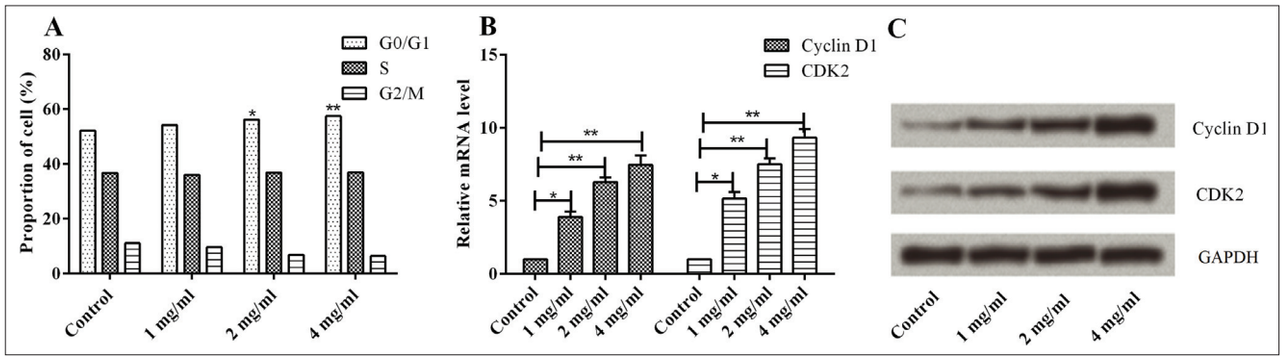

Fig. 3: Effect of ligustrazine on cell cycle. The percentage of cells in G0/G1, S, and G2/M phases (3 A) and cell cycle-related protein cyclin D1 (3 B) and CDK2 (3 C) were determined after administration of different concentrations of Angelica dahurica (1 mg/ml, 2 mg/ml, and 4 mg/ml). The results revealed that the percentage of cells in G0/G1 phase and both mRNA and protein levels of Cyclin D1 and CDK2 were significantly elevated by Angelica dahurica. CDK, cyclin-dependent kinase; $^{*}P < 0.05$ or $^{**}P < 0.01$ compared to the control group

clotrimazole cream by promoting regeneration. The study *in vitro* revealed that the protective role of Angelica dahurica in pressure sores might be associated with increasing cell growth.

Pressure sores, also known as pressure ulcers, decubitus ulcers, or bedsores, are affecting 2 % - 28 % of nursing home residents (Park-Lee and Caffrey 2009). They are an important cause of morbidity, which results in patient disruption and distress, reduction of quality of life and recurrent severe infection (Chou et al. 2015). Currently, the management of pressure sores mainly includes non-surgical interventions (e.g. antimicrobials, nutritional supplementation, dressing, mattress, hydrotherapy, negative pressure therapy, electromagnetic therapy, and solutions) and surgical treatment (e.g. debridement, ostectomy, and musculocutaneous and fasciocutaneous flap closure) (Chantelau 2006; Heyneman et al. 2009; Dissemond 2010; Levine et al. 2012; Cushing and Phillips 2013; Aziz and Bell-Syer 2015). For category I and II ulcers, they are conservatively treated with dressings and the removal of precipitating factors (Hunter and Davies 2014). In China, TCM is collectively and commonly used for prevention and treatment of ulcers for many years. Cured rot and flat sore ointment (CHMO), acupuncture and moxibustion are the three major approaches (Zhang et al. 2013). Although there are different prescription formulations in TCM, the effects of TCM on ulcers could be attributed mainly to the improvement of local blood circulation and acceleration of tissue regeneration (Wang et al. 2012). In the present study, we focused on the effects of Angelica dahurica dressing on category I and II ulcers. Patients with phase I and II pressure sores were registered in our study. Angelica dahurica dressing was externally applied to the patients in the treated group, whereas compound clotrimazole cream was applied to the patients in the control group. In addition to the pharmacotherapy, nursing intervention was also carried out to all patients. Effective nursing care combined with targeted interventions is basic, fundamental, prerequisite, and important to prevent and/or resolve pressure sores, which could significantly reduce the incidence and development of pressure sores. Our results demonstrated that Angelica dahurica dressing significantly improved the total effective rate. Since Angelica dahurica could promote the recovery of pressure sores, we hypothesized that Angelica dahurica might promote regeneration and improve cell growth.

To confirm the hypothesis and explore the underlying mechanism of the protective role of Angelica dahurica in pressure sores, we first analyzed the expression of IL-8, EGF, TGF-β, and VEGF in the tissues of patients. Wound healing is a complex and multicellular process involving three overlapping phases: inflammation, tissue formation, and tissue remodeling (Montesinos et al. 1997). This complex process is carried out and controlled by a complicated signaling network involving a variety of growth factors, cytokines and chemokines (Barrientos et al. 2008). IL-8 is an endothelial cell chemoattractant and an important proangiogenic factor, which plays a critical role in regulating migration of inflammatory cells such as neutrophil and macrophage migration (Koch et al. 1992; Mukaida et al. 1998). TGF-β is a key regulator of fibro-proliferation, and its accumulation in the wound accelerates the proliferative phase of wound healing (Seaton et al. 2015). VEGF and EGF stimulate endothelial cells activation, proliferation and migration, promote angiogenesis, and initiate reepithelialization (Barrientos et al. 2008; Snyder et al. 2016). These results revealed that Angelica dahurica had a clinical effect on pressure sores by promotion of regeneration. In addition, we performed *in vitro* studies using HaCaT cells to explore the effects of Angelica on cell viability and proliferation. The results of MTT and colony formation assay indicated Angelica dahurica could promote the HaCaT cells viability and proliferation. Our results were partly in line with a previous study (Bai et al. 2012). Moreover, we observed that the effects were shown in a dose dependent manner. Low doses of Angelica dahurica (1 mg/ml) had no effects on cell growth, while 2 mg/ml and 4 mg/ml promoted cell viability and proliferation, and the higher dose, the higher the promotive effects.

We next investigated the promotive cell proliferation of Angelica dahurica by analyzing the cell cycle. The results suggested that Angelica dahurica statistically increased the percentage of cells in the G0/G1 phase, as well as upregulating the expression of cell cycle-related protein Cyclin D1 and CDK2. Cyclin D1 is a G1-specific cyclin that promotes restriction point progression during the G1 phase (Mirkovic et al. 2015). CDK2, a member of the cyclin-dependent kinase family of Ser/Thr protein kinases, is a key factor regulating the cell cycle G1 to S transition (Morgan 1995). These results confirmed that Angelica dahurica accelerated cell growth by facilitating transition of the cell cycle.

In conclusion, our results suggest that Angelica dahurica is clinically effective against I-II phase pressure sores. The protective effect may be associated with the promotion of regeneration and cell growth.

4. Experimental

4.1. Subjects

Between January 2013 and February 2015, a total of 98 patients with pressure sores (phase I and II) visiting our hospital were enrolled in this study. Among the 98 patients, 52 cases were male (54 sores) and 46 cases were female (48 sores), aging from 64 to 80 years. The phases of pressure sores were categorized according to the National Pressure Ulcer Advisory Panel (NPUAP) (Black et al. 2007). These patients were randomly assigned to control and treated group (n = 49 in each group). For the control group, there were 25 males and 24 females with an average age of 65.36±2.6 years, and the average duration was 1.86±0.32 months. For the treated group, there were 26 males and 23 females with an average age of 64.68±2.9 years, and the average duration was 1.72±0.46 months. There were 17 cases suffering from advanced cancer, 13 cases had diabetic gangrene, 11 cases experienced heart failure, and 8 cases had other diseases in the control group. There were 18 cases suffered from advanced cancer, 15 cases had diabetic gangrene, 7 cases experienced heart failure, and 9 cases had other diseases in the treated group. The study was approved by the local Ethics Committee and informed written consent was all obtained from the patients.

4.2. Treatment

Basic holistic nursing was performed to all patients. The pressure sites were regularly observed and preventive measures for pressure sores were established. High protein diet was fed and nutritional support was administered if it was necessary. In addition to holistic

nursing, patients in the control group received treatment with compound clotrimazole cream. Patients in the treated group received Angelica dahurica dressing for continuous 4 weeks. Briefly, the wound was disinfected with iodophor and washed with normal saline (NS). The wound was dried by a high flow of oxygen for 5 min, and then treated with Angelica dahurica patch. The Angelica dahurica patch was changed once a week.

4.3. Therapeutic effect

The therapeutic effect was evaluated based on Diagnostic efficacy of Standard TCM Syndrome (Committee 1994). The evaluation criteria include a healing, markedly effective, effective and ineffective. Crusting and scaling were considered as healing. If the wound area was reduced, there were no secretion, and/or granulation tissue grew well, treatment was classified as markedly effective. Effective represents the wound area not enlarged and decreased leakage . If the wound area was increased and move exudate was released treatment was ineffective.

4.4. Enzyme-linked immuno sorbent assay (ELISA) analysis

Tissue samples were collected from all the patients. The samples were maintained in phosphate buffer saline (PBS), ground, and centrifuged. The supernatant was collected and stored at -80 °C. The concentrations of interleukin-8 (IL-8), epidermal growth factor (EGF), transforming growth factor (TGF)-β, and vascular endothelial growth factor (VEGF) were determined using corresponding ELISA Kits (BioSource, San Diego, CA, USA) according to the manufacturer's instructions. Absorbance at at 450 nm was read using a microplate reader (Bio-Rad, Richmond, CA).

4.5. Cell culture and treatment

Human keratinocytes cell line HaCaT cells were purchased from American Type Culture Collection (ATCC; Manassas, VA, USA). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco Inc, Grand Island, NY, USA) containing 10% heat-inactivated fetal bovine serum (FBS, Gibco), 100 U/mL penicillin and 100 U/mL streptomycin (Invitrogen, Carlsbad, CA), and maintained in a humidified incubator at 37 °C and 5% CO_2. Cells were then implanted into 96-well plates and incubated at 37 °C for 24 h. Different concentrations of Angelica dahurica (1 mg/ml, 2 mg/ml, and 4 mg/ml) were added to the cells, meanwhile the cells in the control group were added only with the culture medium.

4.6. Cell viability

The cell viability was assessed by 3-(4, 5-dimethylthiazol-2-yl)-2, 5- diphenyltetrazolium bromide (MTT) assay. Briefly, the cells (5×10^4/mL) were implanted in 96-well plates and washed with PBS. After incubation with different concentrations of Angelica dahurica (1 mg/ml, 2 mg/ml, and 4 mg/ml) for 48 h, 20 µL MTT (0.5 mg/mL) was added to each well and incubated for another 4-6 h at 37 °C. The optical density (OD) of each well was detected at 570 nm optical density (OD) by a microplate reader (Bio-Rad, Richmond, CA).

4.7. Colony formation assay

HaCaT cells were implanted in 12-well plates and then incubated with different concentrations of Angelica dahurica (1 mg/ml, 2 mg/ml, and 4 mg/ml) for 48 h. Thereafter, the cells were washed three times with PBS and cultured in DMEM medium supplemented with 10% FBS for another 7-10 days. The cells were then stained with 0.5% crystal violet at room temperature.

4.8. Cell cycle analysis

HaCaT cells were plated in 96-well plates and incubated with different concentrations of Angelica dahurica for 48 h at 37 °C. The cells were then collected and fixed with cold 70% ethanol at 4 °C overnight. Subsequently, the cells were washed with PBS and incubated with 50 µg/mL propidium iodide (PI) (Sigma-Aldrich, St Louis, MO, USA) with RNase A at room temperature in the dark for 1 h. Afterwards, the percentage of cells in G0/G1, S, and G2/M phases were analyzed using a FACScan flow cytometer (Becton Dickinson, San Jose, CA).

4.9. Quantitative real-time RCR (qRT-PCR)

After incubation with different concentrations of Angelica dahurica (1 mg/ml, 2 mg/ml, and 4 mg/ml), total cellular RNA was extracted from HaCaT cells with the TRIzol Reagent (Life Technologies) following the manufacturer's instructions. First-strand complementary DNA (cDNA) was synthesized using the RevertAid H Minus First Stand cDNA Synthesis kit (Thermo Fisher Scientific). Amplification reactions were performed on an ABI PRISM 7900 HT system (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA, USA) using SYBR Green PCR Master Mix (Applied Biosystems). GAPDH was used as a loading control. All reactions were done in triplicate.

4.10. Western blotting

HaCaT cells were treated with different concentrations of Angelica dahurica for 48 h. Then the cells were harvested, washed with PBS, and lysed with ice-cold cell lysis (RIPA) buffer containing protease inhibitor cocktail (Sigma-Aldrich). The concentrations were determined by Bicinchoninic acid (BCA) Protein Assay Kit (Thermo Scientific, Rockford, IL, USA). Equal amounts of total protein were separated in sodium dodecyl sulfonate (SDS)-polyacrylamide gelelectrophoresis (PAGE) and electroblotted onto polyvinylidenedifluoride (PVDF; Bio-Rad, USA) membranes. Afterwards, the membranes were blocked with 5% bull serum albumin (BSA) in Tris-buffered saline (TBS) containing 0.05% Tween (TBST) 20 for 2 h and incubated with anti-Cyclin D antibody (Cell Signaling Technology, Inc., Beverly, MA, USA) or anti-cyclin-dependent kinase 2 (CDK2) antibody (Cell Signaling Technology) at 4 °C overnight. The membranes were then washed with TBST, incubated with corresponding horseradish peroxidase (HRP)-conjugated secondary antibodies (Cell Signaling Technology) for 2 h at room temperature, and visualized by enhanced chemiluminescence (Pierce, Rockford, IL, USA). GAPDH was used as a loading control.

4.11. Statistical analysis

All experiments were run in triplicate. Data were shown as the mean ± standard ± deviation (SD). Statistical analyses were conducted using SPSS 18.0 (SPSS, Chicago, IL, USA). Student's t-test (for two groups) or one-way analysis of variance (ANOVA) (for more than three groups) was used to compare statistical differences. A statistical significance was set when $P < 0.05$.

Conflict of interest: None declared.

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