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Original Article

Cyaonoside a Promotes Osteogenesis and Fracture Healing by Activating PI3K-AKT Signalling Pathway

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Abstract:

Background: Cyaonoside A (CyA) is an active component in the traditional Chinese medicine Achyranthes bidentata. CyA promotes chondrogenic differentiation of mesenchymal stem cells (MSCs). However, the effect of CyA on osteogenesis and fracture healing, as well as the underlying mechanism has not been investigated.

Materials and Methods: MSCs were isolated from bone marrow of C57 mice. The expression of genes or proteins were detected by real-time PCR or western blot. Open femoral fracture model of mice was used to evaluate the effect of CyA on fracture healing.

Results: In the present study, we found that CyA promoted osteogenesis of MSCs. Then we demonstrated that CyA promoted the phosphorylation of Akt and activated the PI3K-Akt signaling pathway in vitro. Finally, using the mice open femur fracture healing model, we showed that CyA promoted fracture healing in mice. The immunohistochemical result showed that CyA increased the levels of p-Akt, osterix (OSX) and runt-related transcription factor 2 (Runx2) in the fracture callus.

Conclusion: Taken together, our findings suggested CyA promoted osteogenesis and fracture healing by activating the Akt signaling.

Keywords: Cyaonoside A; osteogenesis; fracture; Akt signalling; mesenchymal stem cells; Runx2

Introduction

Fracture is a common clinical disease caused by trauma. In most of the fractures, especially in long bone fractures, how to cut down the period of healing and avoid complications such as reduction failure, delayed healing even non-union is always the overriding problem [1, 2]. Fixation and operation treatment can be the answer, but its limitation may cause an aggravation of the patient's psychological pressure and money [3-6]. Mesenchymal stem cells (MSCs) are the main cells responsible for bone formation, which can be

used to treat bone diseases including fractures, osteoporosis, bone defects, etc. [7-9]. The intrinsic differentiation potentials of MSCs include but are not limited to osteogenesis, chondrogenesis, and adipogenesis in vitro and in vivo [10-12]. These properties of MSCs are indispensable for efficient bone formation and regeneration.

For the regulation of osteogenesis, there is substantial evidence showing many signaling pathways are involved in this process, such as the bone morphogenetic protein (BMP), Wnt, and phosphoinositide 3-kinase (PI3K)/Akt signaling [13, 14]. Akt is the downstream effector of PI3K. The generation of phosphatidylinositol 3,4,5-trisphosphate (PIP3) at the cell membrane by PI3K phosphorylation of phosphatidylinositol 4,5-bisphosphate (PIP2) is a vital process for the Akt signaling. Knockout of Akt negatively affects murine endochondral ossification. There are three isoforms of Akt in mammalian cells, the deletion of both Akt1 and Akt2 in mice leads to severely impaired skin and bone development, and the mice die shortly after birth [15]. The deletion of Akt1 is not lethal, but the secondary ossification is delayed in the long bones and mice also display reduced size compared to the littermates throughout life [16]. Similarly, the specific inhibitor of PI3K/Akt signaling, LY294002, has also been demonstrated to inhibit longitudinal bone growth of mouse embryonic tibiae [17].

The active compounds derived from traditional Chinese medicine has a good curative effect and clinical basis in promoting fracture healing and preventing complications. The regulations of MSCs' cell growth, proliferation, differentiation, and further calcium mineralization by traditional Chinese medicine or natural compounds might be promising strategies for accelerating bone fracture healing [18].

Cyaonoside A (CyA), an active component in the traditional Chinese medicine Achyranthes bidentata, has been shown to promote chondrogenic differentiation of mesenchymal stem cells [19]. Achyranthes bidentata is rich in polysaccharides, steroidal compounds and saponins, and its monomers have been reported to promote bone regeneration, inhibit bone resorption, and have anti-inflammatory and anti-oxidation functions [20-22]. However, there is still a lack of experimental studies on the efficacy and mechanism of CyA on fracture healing. In this paper, the effect of CyA in promoting fracture healing was explored, and the underlying mechanism was also experimentally verified.

Materials and Methods

Culture of MSCs

The mBMSCs were cultured using 2-week-old C57/6J male mice purchased from Guangzhou University of Chinese Medicine Animal Experiment Center. The cells were cultured at 37 ℃ and 5% CO_2 in the cell incubator with saturated humidity. The medium was changed every other day until the adherent cells reached 80-90% confluence to passage. The mBMSCs cells in passages 2-3 were used for further experiments.

Cell viability

MSCs were treated with different concentrations of CyA (0, 0.2, 0.4, 0.6, 0.8, 1.0 μg/ml) for 48h, then MTT assay was performed according to the manufacture's protocol.

Osteogenic differentiation

The mMSCs were treated with osteogenic induction medium (complete medium containing 100 nmol/L dexamethasone, 10 mmol/L β-glycerophosphate, and 0.05 mmol/L L-ascorbic acid-2-phosphate) for 7 or 14 days, then total RNAs were extracted for qPCR detection, or the cells were fixed and stained with 0.5% Alizarin Red S [23, 24].

Quantitive real-time PCR (qPCR)

Total RNA was extracted using NucleoZOL (MNG, Shanghai, China). All operations were performed according to the instructions. RNA was reversely transcribed into cDNA using RT Master Mix (Takara). qPCR was performed using the CFX96 Real-time PCR Detection System (Bio-Rad). The relative quantification of gene expression was normalized to the expression level of GAPDH. All primers used in this research were listed in **Supplementary Table 1**.

Supplementary Table 1. Sequences of primers for real time qPCR.

Gene	*Forward primer sequence (5' to 3')*	*Reverse primer sequence (5' to 3')*

Name		
ALP	GCAAGGGTGAGGAGGGGTA	CCTCTGAAGGCATTTCATAAGCC
Runx2	ATGCTTCATTCGCCTCACAAA	GCACTCACTGACTCGGTTGG
Osterix	AGCGACCACTTGAGCAAACAT	GCGGCTGATTGGCTTCTTCT
GAPDH	TTGAGGTCAATGAAGGGGTC	TCGTCCCGTAGACAAAATGG

Protein extraction and western blot analysis

The mBMSCs were seeded into six-well plates, and treated with final concentration of 0.5μg/ml and 1μg/ml CyA. Cellular proteins were extracted at 48 hours after intervention. Total protein from mBMSCs was transferred to the PVDF membrane (Merck, IPVH00010, Germany) by SDS-PAGE. The membrane was then incubated with primary antibodies at 4°C overnight. The primary antibodies included Phosphorylated-Protein kinase B (p-Akt) (1:1000, Abcam, UK), Akt (1:1000, Abcam, UK), beta-actin (1:1000, Bioworlde, Beijing, China). The membrane was washed and incubated with a secondary antibody for 1h. The immunoreactions were visualized with a hypersensitive ECL chemiluminescence solution (NCM Biotech, Suzhou, China). After exposure, the bands used image J for image analysis.

Animals

Twenty-four 8-week-old C57/6J male mice with SPF level (20 ± 2 g) were obtained from the Center of Experiment Animals of Guangzhou University of Chinese Medicine. The study was approved by the Academic Committee on the Ethics of Animal Experiments of Shenzhen Hospital of Beijing University of Chinese Medicine (approval number: SZLDH2022LSYA-132). Before modeling, the mice were adaptably housed for one week, fed with free water and a standard diet, and fasted one night.

Mouse Open Femoral Fracture

Intraperitoneal injection of 0.3% sodium pentobarbital at a dose of 0.1-0.2ml/10g was used for abdominal anesthesia. Open femoral fracture was established as our published protocol [25]. Penicillin sodium was administered continuously for three days after surgery. The fractured mice were randomly divided into control and CyA groups. A dose of 100 μL CyA decoction (10 μg/ml) was injected into the fracture site every three days after surgery. The mice were humanely sacrificed with an overdose of isoflurane at 4w after femoral fracture.

Micro-CT analysis

The femurs with fracture were freshly dissected, analyzed by Skyscan 1176 Micro-CT scanner (Bruker Micro-CT, Kontich, Belgium) and CT analyzer software. The bone morphometric parameters including bone volume/total volume (BV/TV) and bone mineral density (BMD) of calluses were calculated, according to our published protocols [25].

Histological staining

The femurs were fixed in 10% buffered formalin overnight, decalcified in 10% EDTA, dehydrated, and paraffin-embedded. Sections were cut into 5 μm thickness and subjected to safranin O-fast green and immunohistochemical staining (IHC) [26]. The paraffin sections of the fractured femur tissue of mice were deparaffinized to water, stained with fast green for bone and Safranin O for cartilage, differentiated with 1% glacial acetic acid, dehydrated with gradient alcohol, and sealed with neutral gum. The femoral fracture tissue was observed by a light microscope and photographed. For IHC staining, sections were incubated with 10% FBS at 37℃ for 1h after peroxidase inactivation and antigen retrieval. Then they were incubated with primary antibody overnight at 4℃ and secondary antibody for 1h at 37℃. After counterstaining with hematoxylin, image acquisition was performed. The primary antibodies used included anti-p-Akt (1:100, ab38449, Abcam, UK), anti-osterix (OSX) (1:100, Santa Cruz Biotechnology, sc-393325, US) and anti-runt-related transcription factor 2 (Runx2) (1:100, Santa Cruz Biotechnology, sc-390715, US). The relative intensity was analyzed using Image J software.

Statistical Analysis

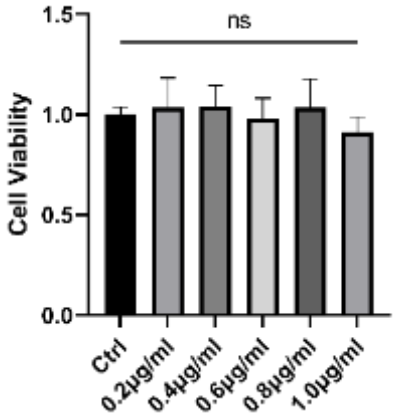
Data were analyzed by GraphPad Prism 9. Quantitative data were represented as mean ± standard deviation (S.D.). Multiple comparisons between groups were statistically analyzed using

one-way analysis of variance (T-test). $P < 0.05$ was regarded as a statistically significant difference.

Results

CyA promotes osteogenesis of MSCs

MSCs were treated with different concentrations of CyA for 48h, then MTT assay was performed to evaluate its cytotoxicity. The result showed that CyA had no cytotoxic effect on MSCs viability (**Supplementary Fig. 1**).



Supplementary Fig. 1. CyA had no cytotoxic effect on cell viability.

After the MSCs were subjected to osteogenic induction for 7 days, the quantitative real-time PCR result showed that the levels of ALP, Runx2 and Osterix were up-regulated in response to CyA (**Fig. 1A**). And the Alizarin Red S staining also confirmed the effect of CyA on osteogenesis of MSCs with more calcium nodules found in the higher concentration group (1.0 μg/ml) (**Fig. 1B**).

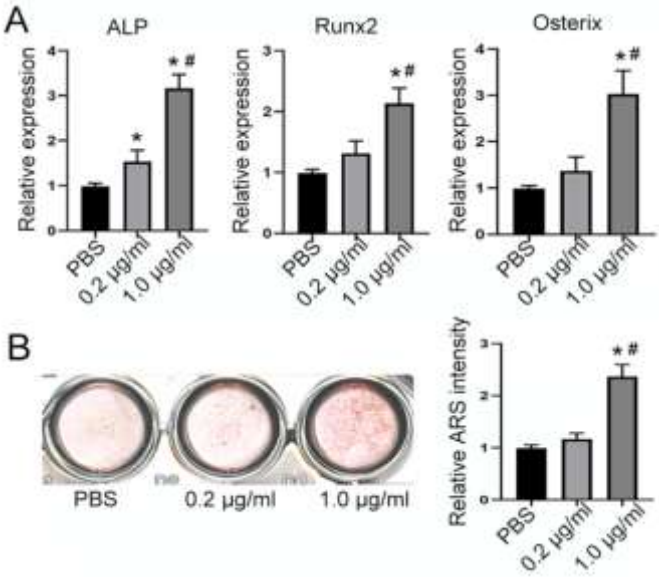


Figure 1 CyA promoted osteogenic differentiation in MSCs. (A) The mRNA levels of ALP, Runx2 and Osterix were checked by quantitative real-time PCR. Data represent mean ± SD (n=5). *p < 0.05, compared to the PBS group. #p < 0.05, compared to the 0.2μg/ml group. (B) Representative images of Alizarin red S (ARS) staining assay.

CyA activates Akt signaling in vitro

Then, western blot was carried out to detect whether Akt signaling was activated or not in CyA-treated MSCs. The result showed that, compared with the control group, the expression of phosphorylated-Akt (p-Akt) was significantly increased by CyA, as well as the ratio of p-Akt to

total Akt (**Fig. 2**), suggesting that CyA promoted osteogenesis of MSCs by activating the Akt signaling.

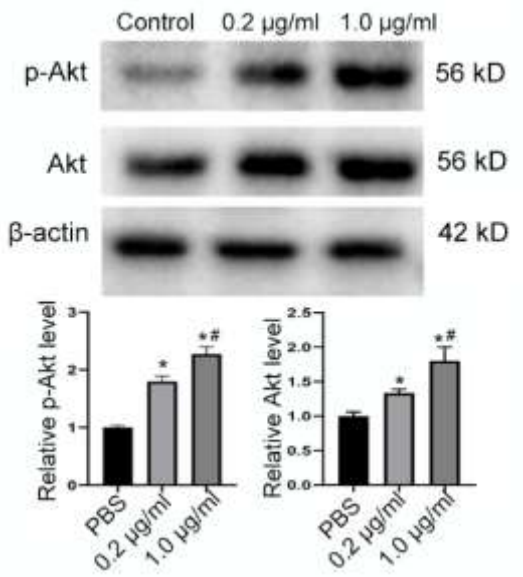


Figure 2 CyA activated Akt signaling pathway. Western blot analysis of p-Akt and Akt. Beta-actin was used as internal control. The relative protein expression levels were quantified by Image J software. Data represent mean ± SD (n=5). *p < 0.05, compared to the PBS group. #p < 0.05, compared to the 0.2μg/ml group.

CyA accelerates fracture healing in mice

At 4w after femoral fracture, the femurs were collected for MicroCT and histological analysis. The results of Micro-CT analysis showed significantly increased BV/TV and BMD in the CyA-treated group (**Fig. 3**).

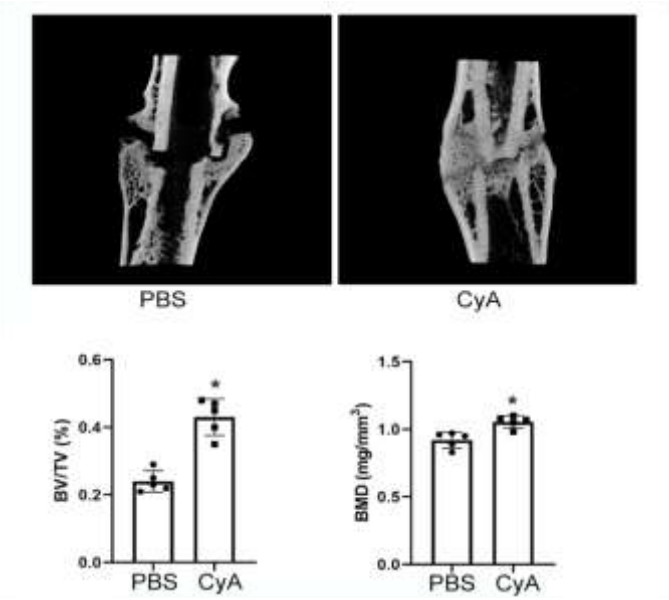


Figure 3 MicroCT analysis of fractured femurs. The fractured femurs in each group were analysed by microCT. Bone histomorphometric parameters including the BV/TV and BMD were calculated. Data represent mean ± SD (n=6), *P <0.05.

The Safranin O-fast green staining result showed that the area of hard callus was higher in the CyA group (**Fig. 4A**). And the IHC staining result showed that CyA treatment significantly increased the expression of Runx2, OCN and p-Akt in the callus (**Fig. 4B**).

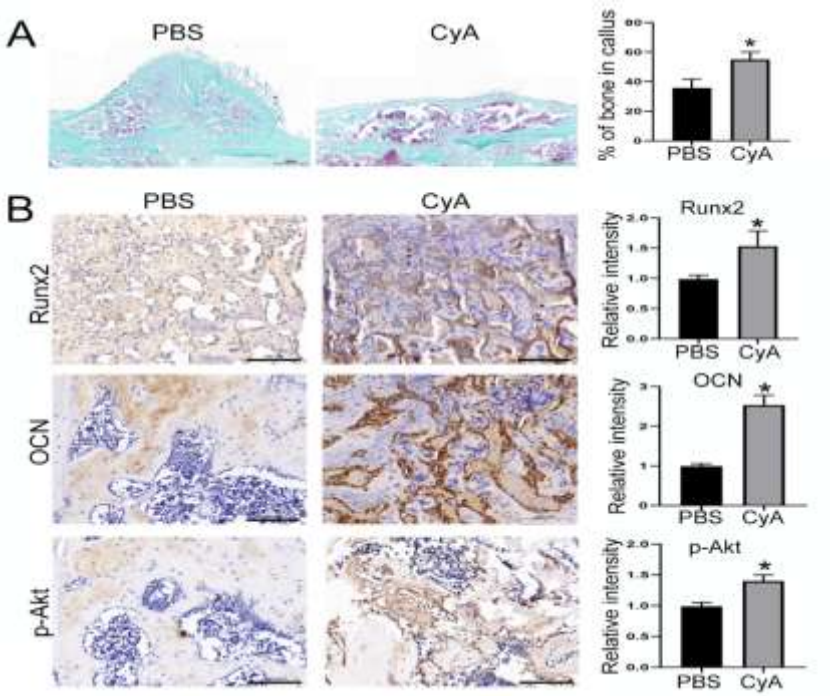


Figure 4 CyA promoted fracture healing In vivo. (A) The femur with fracture of mice was sectioned and stained with safranin O-fast green. Bar scale = 500μm. (B) IHC staining of femoral fractures with indicated antibodies including Runx2, OSX and p-Akt. Bar scale = 100 μm. The relative protein level was quantified with Image J software. Data represent mean ± SD (n=6), *P <0.05.

Discussion

Fracture is a common disease in clinical orthopedics, with a wide distribution of patients, long treatment and rehabilitation time, and accompanied by various complications, which bring a great burden to patients in terms of social activities and personal finances. In this study, we demonstrated that CyA promoted osteogenesis and fracture healing by activating the Akt signaling.

The traditional Chinese medicine (TCM) has been widely used in the treatment of fractures [27-29]. For example, Bin Fang et al have shown Huo Xue Tong Luo capsule can promote osteogenesis of MSCs to ameliorate osteonecrosis of femoral head through inhibiting the transcriptional expression of Miat [30]. Bushen huoxue decoction can inhibit RANKL-stimulated osteoclastogenesis and glucocorticoid-induced bone loss by modulating the NF-κB, ERK, and JNK signaling pathways [31]. Modern clinical studies have shown that the active compounds in TCM have good performance in shortening fracture healing time and suppression of complications, such as quercetin, kaempferol, Baicalein, Coptisine, etc. Quercetin has been found to promote the late healing of fractures in rats, which may be related to its anti-oxidation and maintenance of bone mass [32-34]. Kaempferol could promote the proliferation and differentiation of osteoblasts by activating Wnt/β-catenin pathway in MC3T3-E1 cells, and it has been confirmed by other scholars to effectively maintain bone mass in rats with osteoporosis induced by estrogen [35, 36]. Berberine could reduce the expression of bone degradation enzymes by inhibiting glycogen synthase kinase 3 beta, meanwhile, inhibiting the activity of osteoclasts [37, 38]. Baicalein induced the activation of signaling molecules to inhibit osteoclast formation and promote mature osteoclast apoptosis by inhibiting the receptor activator of NF-κB ligand (RANKL) [39]. Moreover, it could up-regulate the expression of osteoblast differentiation-related markers by stimulating the mammalian target of rapamycin complex 1 signaling pathway [40, 41]. Coptisine has been found to inhibit the maturation of osteoclast precursor cells by inhibiting the phosphorylation of NF-κB p65 and the expression of the key transcription factor NFATc1 induced by RANKL [42].

Previous literature has proved the activation of PI3K-Akt signaling pathway can promote fracture

healing [43, 44]. As a downstream protein of PI3K, Akt can be induced to the cell membrane through the latter, phosphorylate, and activate a variety of downstream signals. Thr308 site is the phosphorylation site of Akt induced by PI3K, which marks the activation of Akt signaling pathway [45]. The deletion of Akt negative affects bone development in mice [15-17], suggesting its key role in bone formation and osteogenesis. Our data also confirmed that CyA could promote osteogenesis by activating the Akt signalling, and several components of CyA has been predicted to be associated with Akt1. However, PI3K-Akt signaling pathway is involved in the regulation of multiple tissues with the crosstalk between different signalling pathways [46-48]. It would be interesting to demonstrate which kind of components account for the activation of Akt signalling, or even the acceleration of fracture healing. More experimental investigation is still needed in the future studies.

Conclusion

Taken together, our study demonstrated that CyA could promote the osteogenesis of MSCs and fracture healing. The underlying mechanism is the PI3K-Akt signaling pathway as confirmed by our in vitro and in vivo results.

Acknowledgement: Not applicable.

Consent for Publication: Not applicable.

Availability of Data and Materials: The data that support the findings of this study are available on request from the corresponding author.

Author Contributions: DTL and LLX conceived, supervised, and commented on all the drafts of this paper. JC, GLH and ZCY conducted the overall experiments and analysis; JLZ and CXH helped in the drafts. All authors read and approved the final manuscript.

Conflict of interests: The authors declare that they have no conflict of interests with the contents of this manuscript.

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