Angelica sinensis polysaccharide promotes the proliferation and osteogenic differentiation of human dental pulp stem cells (hDPSCs) by activating the wnt/β-catenin pathway

Tiantian Mao[1], Youjian Peng[1], Ruobing Peng[1], Xiaoying Wei[2]*

[1]Department of Stomatology, Renmin Hospital of Wuhan University, Wuhan, China; [2]Department of Stomatology, Lishui People's Hospital, Lishui, China

***Corresponding author**: Xiaoying Wei, Department of Stomatology, Lishui People's Hospital, No. 58 Wanfeng Dong Lu, Liandu District, Lishui City, Zhejiang Province, China. Tel: +86-18957093092. Email: weixiaoying11011@163.com

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Abstract

Human dental pulp stem cells (hDPSCs) are capable of forming mineralized nodules. The proliferation and osteogenic differentiation of hDPSCs are very important for alleviating tooth defects caused by related diseases. *Angelica polysaccharide* (ASP) is the main bioactive ingredient extracted from the angelica root. ASP has a variety of biological functions, including immune regulation, antitumor activity, and hematopoiesis. However, its possible effects on hDPSCs are still unclear. In this study, we aimed to investigate the role of ASP in periodontal diseases. We found that ASP promoted the proliferation of hDPSCs and osteogenic differentiation of hDPSCs. We further found that it promoted the expression of osteogenic-related genes, including ALP, RUNX2, Col1a1, and OCN. Mechanically, we found that ASP activated the Wnt/β-catenin pathway. In conclusion, our results suggested that ASP promoted the proliferation and osteogenic differentiation of hDPSCs via the Wnt/β-catenin pathway.

Keywords: *Angelica polysaccharide*; human dental pulp stem cells; osteogenic differentiation; proliferation; Wnt/β-catenin pathway

Introduction

Cell-based tissue engineering is critical in regenerative medicine. Pulp tissues are located in the pulp cavity of teeth. Human dental pulp stem cells (hDPSCs) are capable of forming mineralized nodules (Liu *et al*., 2021). The cells are fusiform, and can self-renew and multidirectionally differentiate (Luan *et al*., 2021b). hDPSCs have attracted increasing attention due to their advantages, such as ease of availability, less immune rejection, and avoidance of ethics (Zhou *et al*., 2019). These excellent functions make hDPSCs suitable sources of tissue repair not only in dentine generation but also in regeneration-related diseases (Zhang *et al*., 2021; Zhou *et al*., 2021). Although the research of hDPSCs has made great progress, the study on hDPSCs is still incomplete. The proliferation and osteogenic differentiation of hDPSCs are very important for alleviating tooth defects caused by related diseases.

The perennial herb Angelica (Oliv. Diels) has been widely used in Asian countries (Nai *et al*., 2021). *Angelica polysaccharide* (ASP) is the main bioactive ingredient extracted from the angelica root (Kwon *et al*., 2021). Most polysaccharides reported in the literature are heteropolysaccharides (Huang *et al*., 2021). Studies have shown that ASP had a variety of biological functions, including immune regulation, anti-tumor activity, and hematopoiesis (Cheng *et al*., 2021; Guo *et al*., 2021). ASP can promote mesenchymal stem cell proliferation and osteoblast differentiation by regulating H19 (Zhu *et al*.,

2021). It could also contribute to the osteogenic differentiation of hDPSCs and the repair of bone defects in type 2 diabetic rats under high glucose state, which may be related to the activation of the Wnt/β-catenin pathway (Song *et al*., 2021). However, its possible effects on hDPSCs are still unclear.

The Wnt/β-catenin pathway is involved in the tooth formation region at every stage of tooth development (Chao *et al*., 2021). The Wnt/β-catenin pathway has been confirmed to play an important role in regulating cell proliferation and differentiation (Luan *et al*., 2021a; Yang *et al*., 2021b). Studies have shown that the Wnt/β-catenin signaling pathway affected the process of osteogenic or adipogenic differentiation of hDPSCs. In addition, classical Wnt/β-catenin significantly promoted apical papilla proliferation and dentin/osteoblast differentiation. Therefore, this pathway could serve as a promising target for the treatment of periodontal diseases.

In this study, we aimed to investigate the role of ASP in periodontal diseases. We confirmed that ASP could promote the proliferation and osteogenic differentiation of hDPSCs via Wnt/β-catenin pathway. Our data therefore confirmed that ASP could serve as a promising drug for the treatment of diseases related to dental pulp.

Materials and Methods

Extraction of ASP

The raw A. sinensis polysaccharide was extracted from the fresh roots of A sinensis (Oliv.) Diels by boiling water extraction and alcohol precipitation method as previously described (Wang *et al*., 2016). The refined polysaccharide, named ASP, was obtained by freeze-drying.

Cell culture

hDPSCs were obtained from the premolars of donors at the age of 12–14 years. All procedures performed in this study involving human participants were in accordance with the standards upheld by the Ethics Committee of Renmin hospital of Wuhan University (Approval no. JZ-1060101) and with those of the 1964 Helsinki Declaration and its later amendments for ethical research involving human subjects. Afterward, the dental pulps were isolated and dispersed with 1 mg/mL type I collagenase (Gibco, USA) at 37°C for 1 h. Then, the tissues were transferred into culturing medium containing low glucose Dulbecco's modified Eagle's medium (DMEM, Gbico, USA), 1% penicillin-streptomycin, and 10% fetal bovine serum (FBS, Gibco, USA), and maintained at 37°C, with the medium changed every 3 days.

CCK-8 assay

To detect cell viability in hDPSCs, CCK-8 assay was performed. Briefly, hDPSCs were plated at 3×10^3 cells per well into a 96-well plate and maintained in complete growth media for 24 h. After indicated treatments, cells were treated with 10 μl CCK-8 solution at 37°C for 1 h. The absorbance of each well was determined with a microplate spectrophotometer at 450 nm (Bio-Rad Laboratories Inc., Hercules, California, USA).

EdU staining

After indicated treatment, cells were fixed with 4% formaldehyde in PBS in each well and permeabilized with 0.5% Triton X-100 in PBS. Then, Click-iT® reaction cocktail was added to each well for 30 min in dark. Remove the reaction cocktail, then wash each well once with 1 mL of 3% BSA in PBS. DAPI was used for nuclear staining.

Alizarin red staining

After indicated stimulation in hDPSCs, Alizarin red staining (ARS) (HY-120601, MedChemexpress, USA) was performed to evaluate mineral deposition. Cells were fixed with 4% paraformaldehyde for 15 min at room temperature, washed thrice with PBS, and stained with 0.5% ARS. Finally, hDPSCs were incubated with 10% cetylpyridinium chloride. Then, the absorbance was detected at 560 nm wavelength via a microplate reader.

Alkaline phosphatase activity

After centrifuging at 1000 g for 10 min, cell pellets were sonicated on ice. Cell lysates were collected and kept on ice for subsequent detection. The protein concentration in the supernatant was determined with a BCA kit (Abcam, UK). Reagent 1 and reagent 2 were sequentially added to the lysates and incubated with cells for 15 min at 37 , and then reagent 3 was added immediately, and vortexed immediately. The OD values of each tube at 520 nm wavelength were acquired with 0.5 cm optical path quartz cuvette.

Immunoblot assay

Proteins were extracted from hDPSCs with RIPA buffer (Beoytime). Then, protein samples were subjected to electrophoresis and transferred onto PVDF membranes. After being blocked with 5% fat-free milk in TBST buffer for 1 h, the membranes were incubated with primary antibodies targeting RUNX2 (1:1000, Abcam, UK), alkaline phosphatase (ALP) (1:1000, Abcam, UK), OCN (1:1000, CST, USA), Wnt3a (1:1000, Abcam, UK), β-catenin (1:1000, Abcam, UK), APC (1:1000, Abcam, UK), cyclinD1 (1:1000, Abcam, UK), and GAPDH (1:10000, Abcam, UK) at 4°C overnight. Then, the membranes were conjugated

with indicated secondary antibodies for 1 h. The membranes were developed with ECL kit (Abcam, UK).

Statistical analysis

Data were displayed as mean ± SD. Statistical analysis was conducted with GraphPad. Significance was assessed by analysis of variance (ANOVA). P < 0.05 was considered statistically significant.

Results

ASP enhances hDPSCs proliferation

After the dissection of hDPSCs, the effect of ASP on cell viability was assessed. As shown in Figure 1A, ASP treatment with varying concentrations induced enhanced cell viability (Figure 1A). Cell proliferation in response to ASP was detected by EdU staining. ASP treatment significantly elevated cell proliferation in hDPSCs (Figure 1B). These data suggest that ASP enhances proliferation of hDPSCs.

ASP could promote osteogenic differentiation of dental pulp stem cells

The effect of ASP on hDPSCs osteogenic differentiation was assessed by ALP activity and ASP staining. ALP activity is used as an early marker of osteogenesis in the bone-forming system. It was increased in ASP-induced hDPSCs in a dose-dependent manner (Figure 2A). ARS staining was conducted for the detection of calcium deposition. ASP stimulation induced more calcium deposition at the concentration of 200 ug/mL (Figure 2B). Thus, these results suggested that ASP promoted osteogenic differentiation of hDPSCs.

ASP promotes osteogenic differentiation in hDPSCs

To further verify the osteogenic effect of ASP on hDPSCs, marker genes in osteogenesis were detected, including RUNX2, ALP, and OCN. ASP treatment significantly enhanced the level of RUNX2, ALP, and OCN, compared with control cells at the concentration of 100 ug/mL.

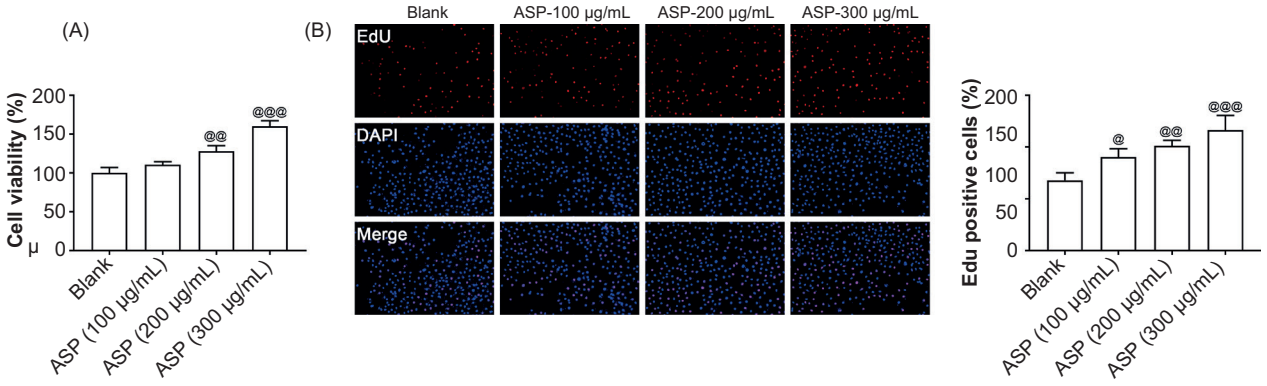

Figure 1. ASP enhances hDPSCs proliferation. (A) Cell viability of hDPSCs treated with increasing concentration of ASP was subjected to CCK-8 assay. (B) EdU staining of hDPSCs treated with increasing concentration of ASP. @, *P* < 0.05; @@, *P* < 0.01; @@@, *P* < 0.001.

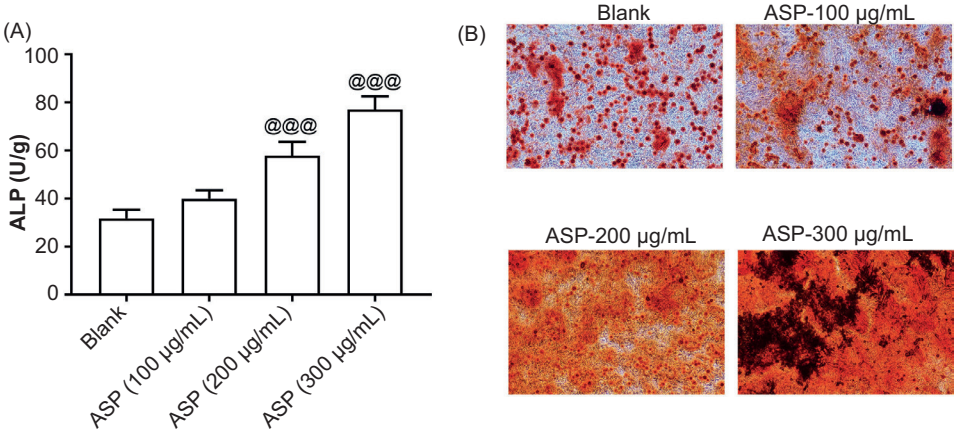

Figure 2. ASP can promote osteogenic differentiation of dental pulp stem cells. (A) Cells treated with ASP were subjected to ALP activity detection. (B) Cells treated with ASP were subjected to calcium deposition detection by ARS. @, *P* < 0.05; @@, *P* < 0.01; @@@, *P* < 0.001.

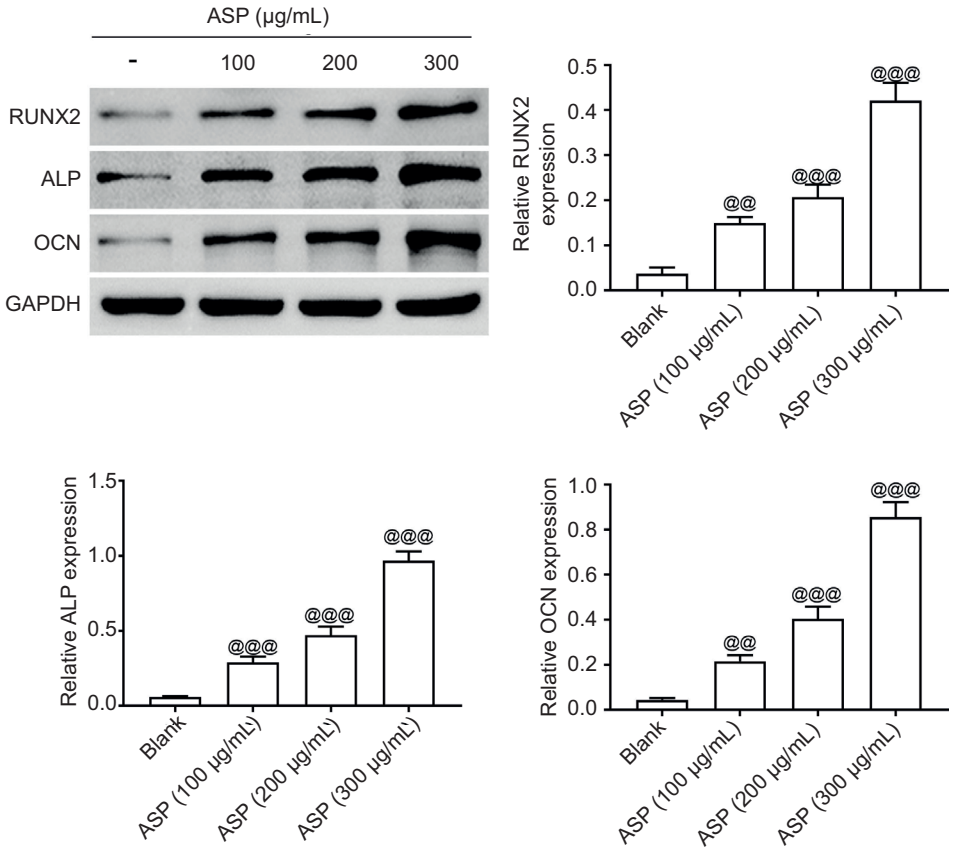

Figure 3. ASP promotes osteogenic differentiation in hDPSCs. The level of RUNX2, ALP, and OCN were detected in cells treated with ASP. @, *P* < 0.05; @@, *P* < 0.01; @@@, *P* < 0.001.

ASP at the concentration of 200 ug/mL and 300 ug/mL further enhanced the protein level of RUNX2, ALP, and OCN (Figure 3). Thus, ASP was proved to induce osteogenic differentiation.

ASP promoted the proliferation and osteogenic differentiation of hDPSCs by activating the Wnt/β-catenin signaling pathway

To investigate the potential mechanism underlying the promoting of proliferation and osteogenic differentiation of hDPSCs, the Wnt/β-catenin signaling pathway was measured. ASP stimulation promoted the level of Wnt3a, β-catenin, and cyclinD1. However, the level of APC was reduced following ASP treatment at the concentration of 100 ug/mL (Figure 4). These data implied that ASP promoted the proliferation and osteogenic differentiation of hDPSCs by activating the Wnt/β-catenin signaling pathway.

Discussion

hDPSCs are fusiform and can self-renew and multidirectionally differentiate. In addition, hDPSCs have attracted more and more attention due to the ease of availability, less immune rejection, and avoidance of ethics (Zhou *et al*., 2021). Therefore, hDPSCs are suitable sources of tissue repair not only in dentine generation but also in regeneration-related diseases, such as diseases related to dental pulp (Liu *et al*., 2020; Zhou *et al*., 2019). Recently, cell-based tissue engineering is widely used in regenerative medicine. In this study, we revealed a promising drug, ASP, which has the potential to affect the proliferation and osteogenic differentiation of hDPSCs. Therefore, our results suggested that ASP could serve as a promising drug for the treatment of diseases related to dental pulp.

By Edu and CCK-8 assays, we noticed that ASP could promote the proliferation of hDPSCs. Furthermore, through ALP and ARS assays, we found that it could contribute to osteogenesis differentiation of hDPSCs. In addition, ASP promoted the expression of osteogenesis genes. Therefore, our results confirmed the key activities of ASP on hDPSCs. The multiple biological activities of ASP in different types of diseases, such as immune regulation, antitumor activity, and hematopoiesis, have been widely revealed (Li *et al*., 2021). ASP alleviated

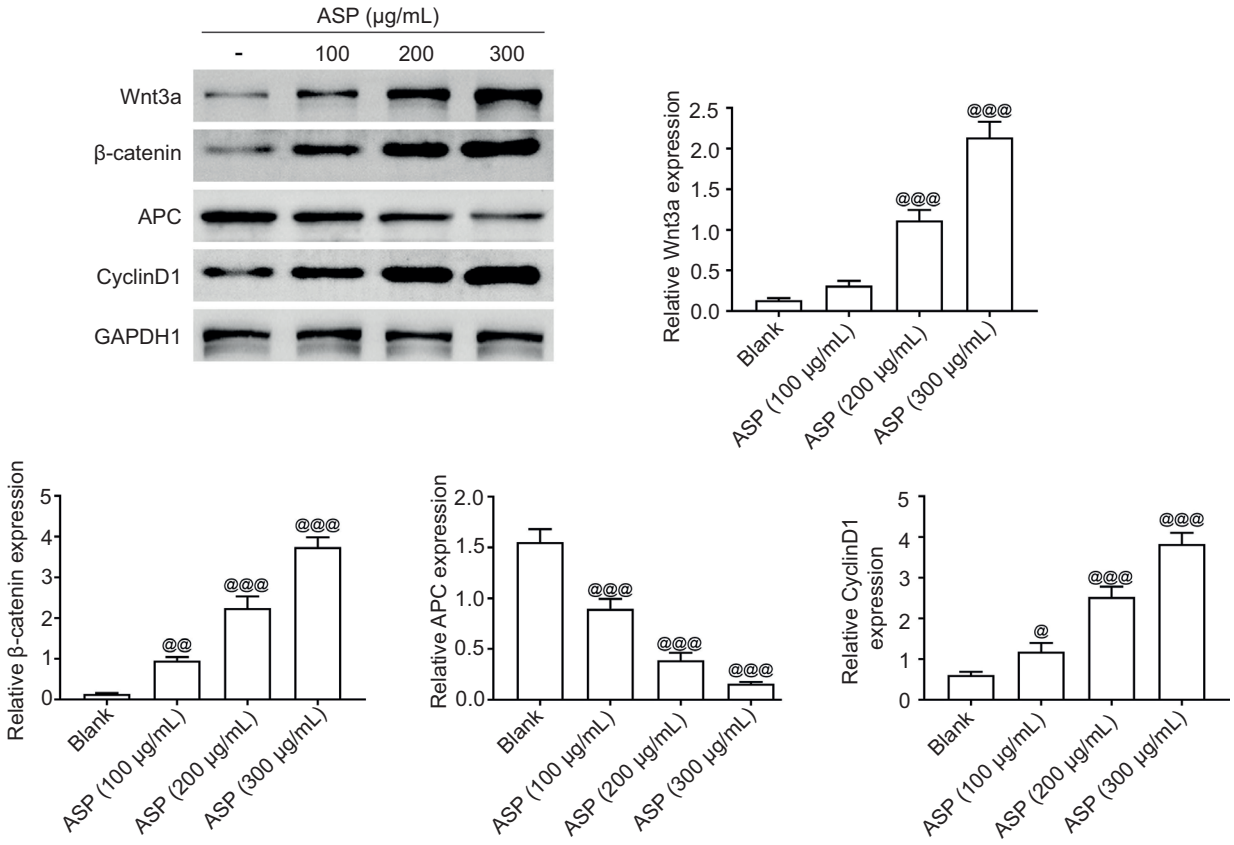

Figure 4. ASP promoted the proliferation and osteogenic differentiation of hDPSCs by activating the Wnt/β-catenin signaling pathway. The level of Wnt3a, β-catenin, and cyclinD1 were detected in cells treated with ASP. @, *P* < 0.05; @@, *P* < 0.01; @@@, *P* < 0.001.

myocardial fibrosis and oxidative stress in the heart of hypertensive rats (He *et al*., 2021). ASP could antagonize 5-FU-induced oxidative stress injury to suppress apoptosis in the liver. ASP attenuated diosbulbin-B-induced hepatotoxicity via the MEK/ERK pathway (Chao *et al*., 2021). ASP also attenuated SNP-induced apoptosis in osteoarthritis chondrocytes by inducing autophagy (Li *et al*., 2021). In this study, we found its effects on the proliferation and osteogenesis differentiation of hDPSCs. These studies confirmed that ASP played key roles in combating multiple types of diseases (Ali *et al*., 2021).

The Wnt/β-catenin pathway is involved in the progression of tooth development. Multiple proteins and drugs mediated tooth development via this pathway (Chang *et al*., 2017). For example, BMP9-initiated osteogenic differentiation of tooth germ mesenchymal cells (TGMCS) in mice required Wnt/β-catenin activity. Runx2 mediated mouse tooth root development via the activation of Wnt/β-catenin pathway (Yang *et al*., 2021c). Sequential stimulation with BMP4 contributed to the differentiation of human embryonic stem cells (hESCs) into dental epithelium via this pathway. These studies, together with our findings, confirmed that the Wnt/β-catenin pathway could serve as a promising target for the treatment of tooth-related diseases.

In addition, the Wnt/β-catenin pathway has proved to play an important role in regulating cell proliferation and differentiation. Previous studies showed that the Wnt/β-catenin signaling pathway affected the process of osteogenic differentiation of hDPSCs (Su *et al*., 2020). In addition, Wnt/β-catenin obviously contributed to apical papilla proliferation and dentin/osteoblast differentiation. miR-330-5p derived from plastrum testudinis-preconditioned bone MSCs could attenuate oteogenesis via this pathway (Yang *et al*., 2021a). Overexpression of HOXB4 could promote the protection of hDPSCs against acute lung injury via the activation of the Wnt/β-Catenin pathway (Tsuruda *et al*., 2021). LincRNA also played a vital role in melatonin-mediated osteogenic differentiation of hDPSCs via targeting this pathway. Similarly, we also found that ASP promoted the proliferation and osteogenic differentiation of hDPSCs via this pathway.

In conclusion, we found that ASP promoted the proliferation of hDPSCs and contributed to osteogenic differentiation. Mechanically, we found that ASP activated the Wnt/β-catenin pathway. In conclusion, our results suggested that ASP promoted the proliferation and osteogenic differentiation of hDPSCs via the Wnt/β-catenin pathway.

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Competing interests

The authors state that there are no conflicts of interest to disclose.

Ethics approval

Not applicable.

Statement of Human and Animal Rights

Not applicable.

Statement of Informed Consent

Not applicable.

Contribution of authors

Tiantian Mao and Youjian Peng designed the experiments, Ruobing Peng carried out the experiments and analyzed and interpreted the data, and Xiaoying Wei prepared the manuscript with contributions from all co-authors.

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