Low Dose *Sophora flavescens* Polysaccharide Promote the Migration and Osteogenic Differentiation of Dental Pulp Stem Cells and Up-Regulate the Expression of Osteogenic Genes

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Yao *et al.*: Effect of *Sophora flavescens* Polysaccharide on Dental Pulp Stem Cells

This work aimed to investigate the effect of *Sophora flavescens* polysaccharide on proliferation and osteogenic differentiation of dental pulp stem cells. The dental pulp stem cells were isolated from rat dental crown tissue and the surface biomarkers were identified by flow cytometry. The osteogenic differentiation of dental pulp stem cells was assessed by alizarin red staining and alkaline phosphatase staining. The dental pulp stem cells were treated with different doses of *Sophora flavescens* polysaccharide for different time points and cell viability was assessed by cell counting kit 8. Cell migration was detected by Transwell and wound healing assay. The expression of collagen I, osteocalcin I, runt-related transcription factor 2 and beta-catenin were measured by quantitative real time polymerase chain reaction assay and Western blotting assay. The cluster of differentiation 90 was highly expressed and cluster of differentiation 45 expressions was relatively low on surface of isolated dental pulp stem cells. *Sophora flavescens* polysaccharide suppressed the proliferation of dental pulp stem cells. The treatment with low dose *Sophora flavescens* polysaccharide (0.2 mg/ml) promoted the migration and osteogenic differentiation of dental pulp stem cells, but higher concentration of *Sophora flavescens* polysaccharide (1 and 10 mg/ml) suppressed the migration and osteogenic differentiation of dental pulp stem cells. Treatment with low concentration of *Sophora flavescens* polysaccharide up-regulated the expression of osteogenic biomarkers. Low dose *Sophora flavescens* polysaccharide can promote the migration and osteogenic differentiation of dental pulp stem cells and up-regulated the expression of osteogenic genes.

Key words: Dental pulp stem cells, *Sophora flavescens* polysaccharide, proliferation, migration, osteogenesis

Oral health is an important indicator for overall health that certified by the World Health Organization (WHO). However, the preservation of complete dental tissues is a tricky issue in clinical studies and application[1]. Dental pulp is the only soft connective tissue in oral cavity that filled with mineralized structures and plays an imperative role in maintaining homeostasis of teeth[2]. The dental pulp tissue is also vulnerable to infection and trauma that may cause irreversible necrosis and pulpitis[3]. Nowadays, the predominant clinical treatment for pulp defects is root canal therapy that basing on pulpectomy and may lead to permanently disabled tooth more susceptible to secondary infection and structural failure[4]. Therefore, profound studies on improving the regenerative technology for dental regeneration to preserve and regenerate the vitality and health of dental pulp is necessary in clinical research. In recent years, increasing efforts in studying stem cell have shaped the perception of their application as pivotal contributors to initiation and development of organs and homeostasis of adult tissue, simultaneously manifesting their specific function in regeneration of damaged tissues that open the gate of novel tissue engineering technics[5,6]. Dental Pulp Stem Cells (DPSCs) is a group of stem cells that found in dental pulp and have been indicated as critical contributor for homeostasis and repair of dental pulp[7,8]. Studies have explored the regulatory mechanisms that composed of extrinsic and intrinsic factors in DPSCs function[9,10]. DPSC based therapeutic strategies, including the preconditioned DPSCs and DPSC aggregates, have indicated particular promising

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future for maintaining oral integrity and health[11]. *Sophora flavescens* (*S. flavescens*) Polysaccharide (SFRP) is a polysaccharide that generated from *S. flavescens*[12,13]. As an extract from traditional Chinese medicine, SFRP exhibits a wild therapeutic spectrum in disease, especially the anti-inflammatory and antioxidative effects[14]. However, the role of polysaccharide from *S. flavescens* in stem cells has not been elucidated yet. In this work, we explored the proliferation, migration and osteogenic differentiation of DPSCs upon SFRP treatment. Our study may provide novel basis to improve the application of DPSCs in tissues regeneration. Sprague-Dawley (SD) rats aged 2 mo old and weighed 250±20 g were brought from Specific Pathogen-Free (SPF) Biotechnology Co., Ltd. All animal experiments were approved and performed according to the guidelines of ethic committee of Xinjiang medical university. The rats were sacrificed and the dental crown was cut open under aseptic conditions. The pulp tissue was cut into 3 small pieces in 1 mm width, followed by digestion with trypsin at 37° for 20 min and placed in culture plate evenly in Dulbecco's Modified Eagle Medium (DMEM) (Gibco, United States of America (USA)) containing 20 % Fetal Bovine Serum (FBS) (Gibco, USA). After the cells form a monolayer, the cells were digested and placed in culture plate. The morphology, size, adherence and growth of the cells were observed under an inverted microscope. Cells were purified by limited dilution. In short, the first generation of rat dental pulp cells at logarithmic growth stage were diluted with high-glucose DMEM containing 10 % FBS at a density of 10-15 cells/ml as single cells and seeded into a 96-well plate with 100 µl/well. After culture for 24 h, the wells with single cell was labeled and added with 200 µl medium. The medium was changed every 5 d at beginning and 3 d when the number of cells increased. The cells were digested and cultured in bigger plate after the confluence. The DPSCs were identified by flow cytometry to check the expression of surface biomarkers Cluster of Differentiation (CD) 45 and CD90. Cell viability was checked by Cell Counting Kit-8 (CCK-8) reagent (Fluorescence, China). In brief, cells were placed into 96-well plate and incubated overnight. Next day, SFRP at different concentrations (0 mg/ml, 0.2 mg/ml, 1 mg/ml, 10 mg/ml, 30 mg/ml, 50 mg/ml, 70 mg/ml and 80 mg/ml) were added into each well and cultured for 1, 3, 5 and 7 d, respectively. At indicated time points, the CCK-8 reagent was added to each well and incubated for another 1 h. The absorbance values at 450 nm were measured by a microplate reader. DPSCs were seeded to 6-well plates and cultured overnight to reach 80 % confluence. Three parallel scratches were created with sterile 200 µl pipette tip. The cells were then washed with Phosphate Buffered Saline (PBS) to remove cell debris and FBS free medium was added for incubation. The images of the wounds were taken at 0, 24 and 48 h after scratching. Cells were resuspended in Minimum Essential Medium (MEM) contains no FBS and placed in the upper chamber of Transwell chambers (5×10^4/well) (Costar, USA). The lower chambers were filled with MEM medium contains SFRP and no FBS. After incubation for 24 h, the chamber was washed by PBS and cells were fixed in 4 % paraformaldehyde for 30 min. The attached cells on the inside of the upper chamber were carefully wiped with cotton swabs. Then cells were stained with 0.2 % crystal violet solution for 10 min and washed with water. The images were taken under a microscope. The DPSCs were placed at a density of 1×10^5/well in 6-well plate and incubated at 37° for 24 h. The osteogenic induction solution (high glucose DMEM+10 % FBS+10 mM β-glycerophosphate+1 µM dexamethasone+50 µg/ml ascorbic acid) was then added and incubated for 21 d. The medium was changed twice every week. After osteogenic induction for 21 d, cells were washed with PBS and stained with alizarin red solution (20 mg/ml) for 5 min. The stained cells were captured under microscope. The Alkaline Phosphatase (ALP) production of DSPCs was checked by using ALP staining kit (SolarBio, China) as per the manufacturer's introduction. The images were captured under microscope. The total Ribonucleic Acid (RNA) was extracted from DPSCs using the TRIzol reagent (Invitrogen, USA) and reverse-transcribed to complementary Deoxyribonucleic Acid (cDNA) by using Super Script III Reverse Transcriptase (RT) kit (Invitrogen, USA) following manufacturer's protocol. The expression of genes was measured by quantitative Polymerase Chain Reaction (qPCR) using SYBR mix (Invitrogen, USA) as per producer's instruction. The RNA levels were normalized to Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) expression. Total proteins were extracted from DPSCs using Radioimmunoprecipitation Assay (RIPA) lysis buffer (MDL, USA) that added with proteinases inhibitor (MDL, USA) and quantified using Bicinchoninic

Acid (BCA) kit (MDL, USA). The equal amounts of proteins were separated in Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) gel and transferred to Polyvinylidene Difluoride (PVDF) membranes. The targeted proteins were then probed by primary antibodies against collagen-I (Affinity, USA), Osteocalcin (Affinity, USA), Runt-Related Transcription Factor 2 (RUNX2) (Affinity, USA) and beta (β)-catenin (Affinity, USA) overnight at 4°. The next day, after incubation with secondary antibodies and reaction with Enhanced Chemiluminescence (ECL) reagent, the blots were visualized in a gel image system. Data in this work were presented as mean±Standard Deviation (SD) of three replicates and analyzed by using Statistical Package for the Social Sciences (SPSS) 20.0 software and GraphPad Prism software. The differences between multiple groups were analyzed using one-way Analysis of Variance (ANOVA) analysis. $p<0.05$ was regarded as significant difference. The morphology of isolated rat DPSCs was observed under microscope. The isolated cells presented regular spindle shape (fig. 1A). The surface biomarkers of stem cells, namely CD90, was highly expressed in isolated DPSCs, whereas the level of CD45 was low (fig. 1B), which indicated the feature of stem cells. Next, we evaluated the effect of SFRP on DPSCs proliferation. The SFRP at high concentrations (30 mg/ml, 50 mg/ml, 70 mg/ml and 80 mg/ml) almost completely suppressed cell proliferation after treatment for 12 h (fig. 1C). Here, we choose the low dose of 0.2 mg/ml, 1 mg/ml and 10 mg/ml for long-term treatment and observed that SFRP suppressed cell proliferation at time- and dose-dependent manner as shown in fig. 1D. To determine the migration of DPSCs, we performed Transwell assay and wound healing assay. As shown in fig. 2A, the SFRP at 0.2 mg/ml could enhance the migration of DPSCs, whereas the higher doses of 1 mg/ml and 10 mg/ml notably suppressed cell migration through the Transwell membrane. The results from wound healing assay confirm this effect. SFRP at 0.2 mg/ml significantly suppressed the healing of scratched wounds and 10 mg/ml SFRP suppressed the wound healing ability of DPSCs as shown in fig. 2B. Subsequently, we evaluated the osteogenic induction effect of SFRP. The formation of calcium nodules was identified by alizarin Red staining and ALP is staining. As shown in fig. 3A and fig. 3B, the SFRP at 0.2 mg/ml enhanced the calcium accumulation in DPSCs, but 1 mg/ml and 10 mg/ml SFRP notably decreased the staining. Moreover, we observed elevated RNA and protein expression of osteogenesis biomarkers, including collagen I, osteocalcin I, RUNX2 and the Wnt signaling effector β-catenin, under 0.2 mg/ml SFRP treatment, which was significantly decreased by higher doses of SFRP (fig. 3C and fig. 3D). The teeth health is closely correlated with the homeostasis of tooth germ tissue, which have complicated regulatory mechanisms, diverse structure and critical functions[15]. The special features of oral structure make it complex to realize tooth regeneration[15]. In recent years, the fast development in the technologies and theories in tissue engineering field have greatly boosted the new

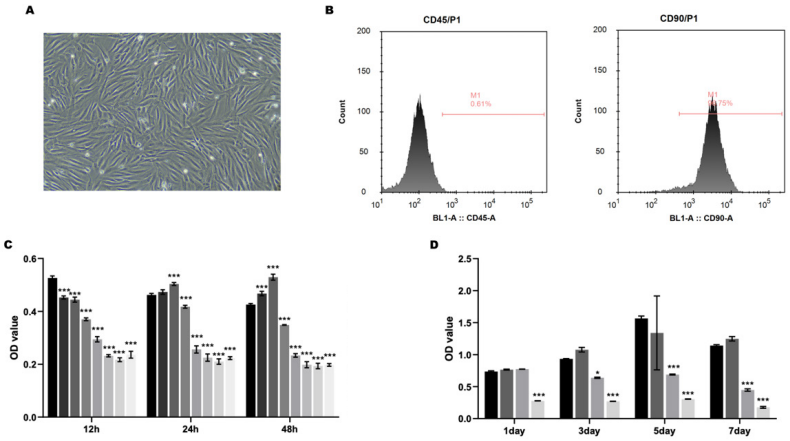

Fig. 1: Identification of DPSCs, (A): The morphology of isolated DPSCs; (B): The CD45 and CD90 on DPSCs were checked by flow cytometry and (C and D): DPSCs were treated with SFRP at different concentrations and time points, and then the cell viability was measured by CCK-8 experiment

Note: *p<0.05, **p<0.01 and ***p<0.001, (C): (■): 0 mg/ml; (■): 0.2 mg/ml; (■): 1 mg/ml; (■): 10 mg/ml; (■): 30 mg/ml; (■): 50 mg/ml; (■): 70 mg/ml and (■): 80 mg/ml, and (D): (■): 0 mg/ml; (■): 0.2 mg/ml; (■): 1 mg/ml and (■): 10 mg/ml

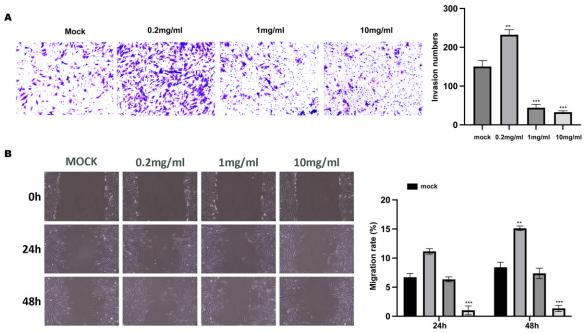

Fig. 2: SFRP affects the migration of DPSCs. DPSCs were treated with SFRP at different doses for 24 h, (A): Transwell and (B): Wound healing assay was performed to measure cell migration
Note: **p<0.01 and ***p<0.001, (■): Mock; (▭): 0.2 mg/ml; (▭): 1 mg/ml and (▭): 10 mg/ml

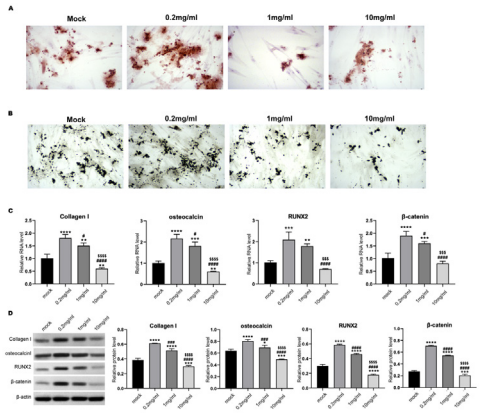

Fig. 3: SFRP induces osteogenic differentiation of DPSCs. MSCs were treated with SFRP for 24 h, (A): Alizarin red staining; (B): ALP staining of calcium in DPSCs; (C): RNA and (D): Protein levels of collagen I, osteocalcin I, RUNX2 and β-catenin in DPSCs
Note: *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 *vs.* mock; #p<0.05, ##p<0.01, ###p<0.001, ####p<0.0001 *vs.* 0.2 mg/ml and $p<0.05, $$p<0.01, $$$p<0.001, $$$$p<0.0001 *vs.* 1 mg/ml

methods for tooth and tissue regeneration[16,17]. The exploration and use of stem cells have greatly speeded the development of tissue engineering area[18]. Stem cells could be isolated from several tissues, including the bone marrow, adipose and dental tissue[19]. Studies have pointed out the tissue regenerative function of DPSCs[20]. Achieving and maintaining reliable and safe lineage specific differentiation of stem cells is important for improving the therapeutic potential of DPSCs, as well as the clinical translation of tissue engineering strategies[21,22]. The dried roots of *S. flavescens* is traditionally used as antipyretic medicine to reduce inflammation[23,24]. The alkaloids and flavonoids are the main constituents of *S. flavescens* and the purified extracts have been widely studied in multiple diseases[25,26]. The ethyl acetate extracts of residues of *S. flavescens* could inhibit the xylene induced mouse auricle edema and carrageenan-induced hind paw edema *in vivo* and lower the production of pro-inflammatory cytokines Tumor Necrosis Factor-Alpha (TNF-α), Interleukin-6 (IL-6), Nitric Oxide (NO) and Monocyte Chemoattractant Protein-1 (MCP-1) in Lipopolysaccharide (LPS) induced *in vitro* RAW264.7 model[27]. Moreover, *S. flavescens* polysaccharide effectively suppressed the H22 tumor growth in mouse model and promotes the splenocyte proliferation, thus resulting in a prolonged life survival of mice. The *S. flavescens* polysaccharide notably strengthened peritoneal macrophages to produce NO *via* stimulating activity of inducible NO Synthase (iNOS) and induce the clearance of tumor cells by macrophages[28]. In this work, we found that SFRP at different concentrations exert diverse effects on the phenotype of DSPCs. We found that higher concentration of SFRP (1 mg/ml and 10 mg/ml) caused inhibited cell proliferation and migration, as well as suppressed the osteogenic differentiation of DPSCs. In contrast, the low dose of 0.2 mg/ml SFRP facilitated the cell proliferation, migration and osteogenic differentiation of DPSCs. These results suggested that treatment with low dose SFRP may improve the regenerative activity of DPSCs. To conclude, we explored the effects of SFRP on DPSCs and determined the appropriate concentration of SFRP that facilitate the proliferation, migration and osteogenic differentiation of DPSCs. Our findings

provided novel theoretical evidence for the better application of DPSCs in the tissue engineering.

Conflict of interests:

The authors declared no conflict of interests.

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