Effects of total flavonoids from *Drynaria fortunei* on the proliferation and osteogenic differentiation of rat dental pulp stem cells

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Abstract. Dental pulp stem cells (DPSCs) have the potential to form bone, nerve and fat, and are a candidate for use in regenerative medicine. Previous studies indicated that total flavonoids from *Drynaria fortunei* show a stimulative effect on the proliferation and osteogenic differentiation of osteoblastic MC3T3-E1 cells *in vitro*. This study aimed to investigate the effect of total flavonoids from *Drynaria fortunei* on the proliferation and osteogenic differentiation of rat DPSCs, and to further clarify the mechanisms involved. DPSCs were isolated by enzymatic digestion and identified using the CD44, CD29 and CD34 markers by immunohistochemistry, and exposed to 0.01, 0.05 and 0.1 g/l total flavonoids from *Drynaria fortunei* media. Total flavonoids from *Drynaria fortunei* promoted the proliferation of DPSCs in a dose-dependent manner and this effect may depend on the shortening of the G0/G1 phase and promotion of the S phase. Compared with the control group, the levels of alkaline phosphatase (ALP) and the expression of osteogenic genes increased with the concentrations of total flavonoids from *Drynaria fortunei*, and the volume and number of calcified nodules in the *Drynaria* groups was bigger compared to the control group. These results suggest that total flavonoid from *Drynaria fortunei* directly stimulates DPSC proliferation and osteogenic differentiation, and may serve as a new promising candidate drug for dental tissue engineering and bone regeneration.

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Introduction

Periodontal disease is a chronic infectious disease and often leads to periodontal tissue defects such as tooth loss and alveolar bone absorption. The periodontal tissue defects often cause the functional decline of teeth, which seriously affects health and quality of life (1). The goal of therapy for destructive periodontal disease is the regeneration of the attachment apparatus. Although investigators have developed methods to treat periodontal disease, the new and effective approaches require improvement for reconstructing periodontal tissues. The technology of periodontal tissue engineering, which is composed of three important elements, such as seeding cells, biomaterials and drugs, is one of the current controversial issues.

Selection of ideal seeding cells is the basic aim of all tissue engineering research. Gronthos *et al* were the first to identify and isolate dental pulp stem cells (DPSCs) (2), and Grottkau *et al* demonstrated that DPSCs were undifferentiated mesenchymal stem cells in tooth pulp with self-renewal, regulated cell proliferation and the potential to transdifferentiate into various types of specialized tissue cells including osteoblast, adipocyte, neurocyte, chondrocyte and smooth muscle cells (3). Findings of recent studies suggested that DPSCs have the ability to differentiate into the bone cell phenotype *in vitro* and form mineralized tissue *in vivo* (4,5). These results suggest that DPSCs are likely have a positive impact on periodontal tissue engineering and bone regeneration.

Another strategy for tissue engineering is the addition of a drug that enhances the efficacy of seeding cells. *Drynaria fortunei* is a common type of traditional Chinese herb in the area of orthopaedics and traumatology, which may be beneficial to bone healing, particularly the total flavonoid. More investigators have begun to pay attention to basic pharmacological and clinical studies on the total flavonoid of *Drynaria fortunei*. Wang *et al* reported that eleven flavonoids from *Drynaria fortunei* exhibited proliferative activity in the UMR106 osteoblastic cell line *in vitro* (6). Jeong *et al* also demonstrated that *Drynaria fortunei* is capable of promoting osteoblastic differentiation and mineralization in osteoblastic MC3T3-E1 cells through the regulation of bone morphogenetic protein-2, alkaline phosphatase (ALP), type I collagen

and collagenase-1, indicating that it has anabolic effects on bone (7). Shu *et al* reported that total flavonoid from *Drynaria fortunei* promotes the osteogenic differentiation of bone mesenchymal stem cells (8).

No study has reported the effect of *Drynaria fortunei* on DPSCs. Thus, in this study we investigated whether total flavonoid from *Drynaria fortunei* regulates the proliferation and osteogenic differentiation of DPSCs for periodontal tissue engineering.

Materials and methods

Cell isolation and culture. Rat DPSCs were isolated enzymatically as previously described (2,9). Sprague-Dawley rats (no limit of male or female, SPF, 25-30 days old, 80-100 g) were provided by the Experimental Animal Center of Tongji Medical College, Huangzhong University of Science and Technology. Animals used in this study were maintained in accordance with the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health and the Policy of Animal Care and Use Committee of General Hospital of the Yangtze River Shipping, Wuhan, China. In brief, the rats were anaesthetized using phenobarbital intraperitoneally. The rat oral cavity was disinfected with 75% alcohol and a cavity of 1.5 mm depth was created in the distal half of the occlusal surface using a high-speed dental handpiece. The pulp tissue was separated from the pulp chamber by penetration with the tip of a sterile dental explorer, and digested in a solution of 3 mg/ml collagenase type I (Biosharp, St. Louis, MO, USA) and 4 mg/ml dispase (Worthington Biochemical Co., Lakewood, NJ, USA) for 1-1.5 h at 37˚C. Dental pulp cell suspensions were seeded in a culture dish of 35 mm in diameter, (Costar, New York, NY, USA) with low-glucose Dulbecco's modified Eagle's medium (DMEM, Hyclone, Logan, UT, USA) supplemented with 10% defined fetal bovine serum (FBS, Sijiqing, China) and 100 U/ml penicillin and 100 μg/ml streptomycin, and then cultured at 37˚C in 5% CO_2. After culture for 14 days, a section of the dental pulp cells formed colonies. The cells in colonies were selected and monoclonal expansive culture was used to obtain DPSCs. DPSCs were digested by 0.25% trypsin and passaged *in vitro*.

Identification of DPSCs by immunocytochemistry. Immunocytochemistry was performed according to the technique described by Almushayt *et al* (9). When cells have grown on glass coverslips, the DPSCs were fixed with 4% paraformaldehyde at room temperature for 30 min. The coverslips were air dried and blocked with 10% goat serum. The coverslips were incubated at a dilution of 1:200 of the primary antibody (rabbit anti-rat polyclonal antibody of CD44, CD29 and CD34; Boster, China) overnight at 4˚C in a humidified chamber. Following incubation, the coverslips were again incubated with anti-rabbit biotinylated IgG for 45 min at room temperature. After the coverslips were incubated with streptavidin peroxidase reagent for 1 h at room temperature, they developed a brown color by using diaminobenzidine (DAB, Boster). The nuclei were counterstained by hematoxylin (Boster). Coverslips were analysed by light microscopy (CKX31, Olympus, Japan).

Preparation of osteogenic medium and drugs. Osteogenic medium was the DMEM/F12 medium (Hyclone) supplemented with 10% FBS, 10^{-8} mM dexamethasone (Sigma, St. Louis, MD, USA), 10 mM β-sodium glycerophosphate (Sigma), 50 mg/l vitamin C (Sigma). Powdered total flavonoid from *Drynaria fortunei* (0.25 g) (Qihuang Pharmaceutical Investment and Research Company, China) was dissolved in osteogenic media and different concentrations of 0.01, 0.05 and 0.1 g/l were yielded.

DPSC proliferation analysis using colorimetric 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. DPSCs were placed into 96-well plates at a density of $2x10^4$ cells per well and maintained overnight. The DPSCs were then treated with medicated media at various concentrations of the total flavonoid from *Drynaria fortunei* (0.01, 0.05 and 0.1 g/l) in a volume of 100 μl. At each time-point, 20 μl of MTT solution (5 mg/ml, Sigma) was added to each well and incubated with DPSCs for 4 h at 37˚C. The MTT media were removed and 150 μl dimethyl sulfoxide (DMSO) was added in all wells in order to dissolve the blue formazan products in DPSCs. The plates were then read using an enzyme-linked immunosorbent assay microplate reader (Sunrise, Tecan, Switzerland) at a wavelength of 570 nm.

DPSC cell cycle analysis using propidium iodide (PI) assay. Following treatment of total flavonoid from *Drynaria fortunei*, DPSCs were collected using trypsinization. DPSCs were resuspended and fixed in cold 70% alcohol for 2 h at 4˚C. DPSCs were then rinsed with PBS and resuspended with PI solution (0.5 mg/ml, Sigma) for 30 min at 37˚C. The samples were analyzed using flow cytometry (FACSCanto II, BD, USA).

ALP activity analysis. Following cell suspension (500 μl) containing 10^4 DPSCs per well was added into 24-well plates and incubated for 48 h, the medicated media with total flavonoid from *Drynaria fortunei* at concentrations of 0.01, 0.05 and 0.1 g/l was added to the well. After culturing for 6 days, the DPSCs were washed with PBS, incubated in 100 μl of 0.2% Triton-100 buffer at 4˚C overnight and centrifuged. Protein concentration of the clear supernatant was determined by the protein quantitative determination kit (Boster). The clear supernatant was used for the measurement of ALP activity using the ALP activity kit (Nanjing Jiancheng, China). ALP activity was read using an enzyme-linked immunosorbent assay microplate reader (Sunrise) at 405 nm.

Evaluation of calcium nodules using Alizarin Red S staining. Cell suspension (2 ml) containing 10^5 DPSCs per well was added into 6-well plates and incubated for 48 h. The medicated media with total flavonoid from *Drynaria fortunei* at concentrations of 0.01, 0.05 and 0.1 g/l was then added in the well. After culturing for 21 days, the DPSCs were rinsed with PBS followed by fixation with 74% paraformaldehyde at room temperature for 30 min and stained with 40 mM Alizarin Red S (Sigma) at 37˚C for 30 min. The DPSCs were then rinsed twice with PBS and visualized under an inverted microscope (CKX31, Olympus, Japan).

Analysis of osteogenic genes using quantitative reverse transcription polymerase chain reaction (qRT-PCR). Following DPSC culturing with medicated media with total flavonoid

Table I. The primer sequences used for qPCR.

Gene	Forward (5'-3')	Reverse (5'-3')
GAPDH	GAAGGGCTCATGACCACAGT	GAAGGGCTCATGACCACAGT
ALP	GCTTCACGGCATCCATGAG	GAGGCATACGCCATGACGT
Osteocalcin	GGCTTCCAGGACGCCTACA	CATGCCCTAAACGGTGGTG
Collagen I	TGGAATCTTGGATGGTTTGGA	GCTGTAAACGTGGAAGCAAGG
Runx-2	CGGAGCGGACGAGGCAAGAG	AGAGTCATCAAGCTTCTGTCTGTGC

GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ALP, alkaline phosphatase.

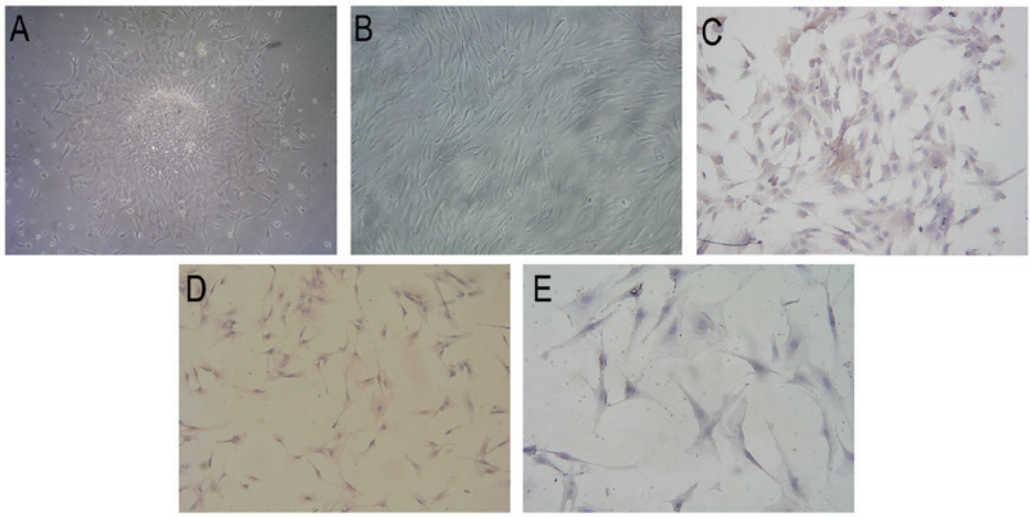

Figure 1. Separation and identification of DPSCs (x100). (A) Dental pulp cells, (B) DPSCs, (C) positive expression of CD44, (D) positive expression of CD29, (E) negative expression of CD34. DPSCs, dental pulp stem cells.

from *Drynaria fortunei* at concentrations of 0.01, 0.05 and 0.1 g/l for 5 days, total RNA was extracted from each sample by TransZol (Transgen, China). The concentration and purity of each RNA sample was measured using a nucleic acid quantitative instrument (NanoDrop ND-100, Thermo, Wilmington, DE, USA). First-strand cDNA synthesis of each sample was performed from 1 μg of total RNA by reverse transcription using the TranScript First-Strand cDNA Synthesis SuperMix (TransGen) and T3000 PCR Amplifier (Biometra, Göttingen, Germany). The reaction conditions were 42˚C for 30 min and 85˚C for 5 min. TransStart Green qPCR SuperMix (TransGen) and StepOne Plus qPCR system (ABI, Carlsbad, CA, USA) were used to perform qPCR. Reaction conditions were an initial denaturation step of 95˚C for 30 sec, followed by 40 cycles of 95˚C for 5 sec, annealing 55˚C for 15 sec and extension 72˚C for 10 sec. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a housekeeping gene to normalize RNA expression, and the gene expression in each sample was calculated by the comparative quantitative method. Each sample was assessed in triplicate. Primer sequences are listed in Table I (10).

Statistical analysis. Data were presented as the means ± standard deviation (SD). Statistical analysis between groups in this study was evaluated using ANOVA to calculate P-values. Differences were considered significant when $P<0.05$.

Results

Isolation and identification of DPSCs. The pulp cells obtained via enzyme digestion were attached to the base of 6-well plates following cell incubation for 24 h. The pulp cells were expanded to fibroblast-like growths, and a section of pulp cells grew rapidly and formed small cell clones after cultivation for 72 h. Following cultivation for 11-13 days, the number and size of the cell clones were significantly increased (Fig. 1A). DPSCs obtained from the pulp cells by clone picking tended to be fusiform and tightly packed when viewed by microscopy (Fig. 1B). Immunocytochemistry was used to explore the expression of stem cell surface markers in DPSCs. The results showed that rat DPSCs were positive for CD44 and CD29, but negative for CD34 (Fig. 1C-E).

Effect of total flavonoid from Drynaria fortunei on DPSC proliferation and cell cycle. The MTT test showed that total flavonoid from *Drynaria fortunei* promoted rat DPSC proliferation in a dose-dependent manner (Fig. 2A). PI staining analysis showed that total flavonoid from *Drynaria fortunei*

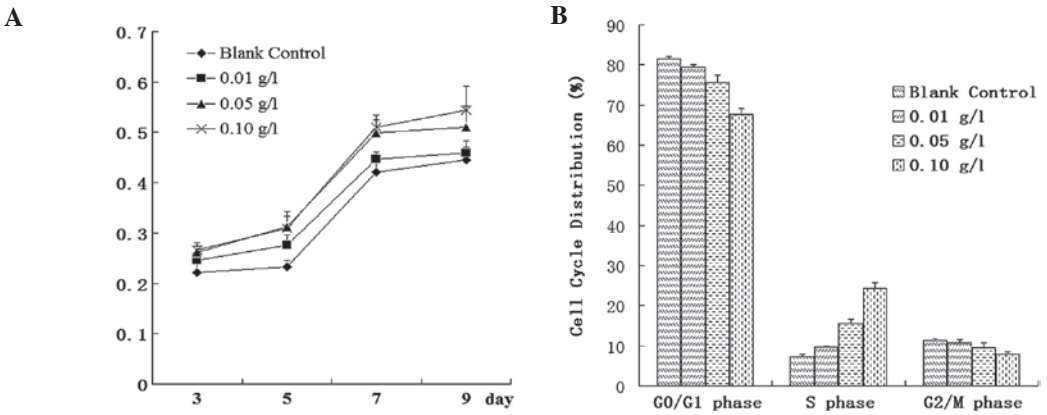

Figure 2. (A) Growth curve of DPSCs after treatment with total flavonoid from *Drynaria fortunei*. (B) Cell cycle distribution of DPSCs after treatment with total flavonoid from *Drynaria fortunei*. DPSCs, dental pulp stem cells.

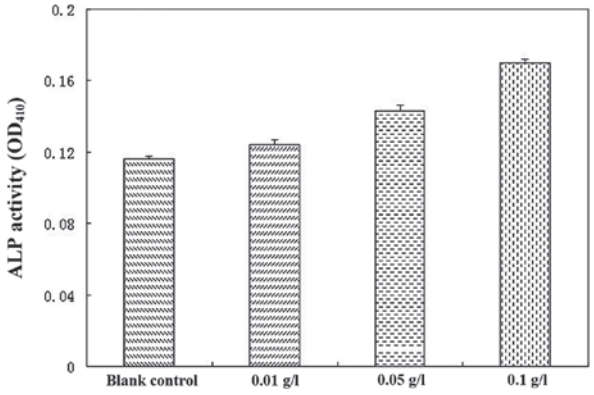

Figure 3. ALP activity of DPSCs following treatment with total flavonoid from *Drynaria fortunei*. ALP, alkaline phosphatase; DPSCs, dental pulp stem cells.

prompted DPSCs to enter into the S phase, which indicates that DPSCs in the S phase increased in a dose-dependent manner, while DPSCs in the G0/G1 phase decreased in s dose-dependent manner. The number of DPSCs in the S phase decreased in the 0.05 and 0.10 g/l groups, but not in the 0.01 g/l group (Fig. 2B).

Effect of total flavonoid from Drynaria fortunei on the ALP activity of DPSCs. We examined the effect of total flavonoid from *Drynaria fortunei* on ALP activity, which is an early marker of osteogenic differentiation. Fig. 3 shows that total flavonoid from *Drynaria fortunei* dose-dependently increased the ALP activity of rat DPSCs to 1.06-, 1.23- and 1.47-fold the blank control activity at 0.01, 0.05 and 0.1 g/l, respectively.

Effect of total flavonoid from Drynaria fortunei on the formation of calcium nodules in DPSCs. Alizarin Red S staining was performed to demonstrate calcium nodules, and the percentage of calcium nodules to total area was found to be significantly higher in the medicated media groups than in the blank control group. Total flavonoid from *Drynaria fortunei* increased the density of calcium nodules compared with the blank control in a dose-dependent manner (Fig. 4). These results suggest that total flavonoid from *Drynaria fortunei* significantly promotes DPSC maturation and the formation of calcium nodules by rat DPSCs.

mRNA expression of osteogenic genes following treatment with total flavonoid from Drynaria fortunei. The levels of ALP, osteocalcin, collagen I and Runx-2 were determined to evaluate the effects of total flavonoid from *Drynaria fortunei* on osteogenic differentiation-related gene expression (Fig. 5). Following exposure to 0.01 g/l total flavonoid from *Drynaria fortunei*, the relative amount of ALP expression was enhanced by 1.2-fold. Total flavonoid from *Drynaria fortunei* at 0.05 and 0.10 g/l markedly elevated the expression of ALP mRNA by 1.35- and 1.37-fold in rat DPSCs. Similarly, exposure of rat DPSCs to total flavonoid from *Drynaria fortunei* induced the expression of osteocalcin, collagen I and Runx-2 in a concentration-dependent manner. Notably, the mRNA expression of collagen I and Runx-2 were found to be upregulated 2.03- and 2.50-fold by total flavonoid from *Drynaria fortunei* at a concentration of 0.10 g/l.

Discussion

Tissue engineering utilizing DPSCs has the potential for dental tissue and tooth regeneration, and to stimulate bone tissue growth and repair. As a first step towards the goal of successful development of tissue engineering in experimental and clinical dentistry, we attempted to explore the effect of total flavonoids from *Drynaria fortunei* on proliferation and osteogenic differentiation in rat DPSCs. We demonstrated that the total flavonoids from *Drynaria fortunei* may be feasible for periodontal tissue engineering and DPSCs have a definite potential for biomedical applications.

The majority of studies was carried out recently on the foundation of primary dental pulp cell cultivation. The methods of primary dental pulp cell isolation included enzyme digestion, explant culture technique and explant digestion. We selected explant enzyme digestion to isolate dental pulp cells, as this method could obtain a suitable amount of living dental pulp cells. Dental pulp is loose connective tissue with high vascularization, and is composed of different cells, such as dental pulp fibroblasts, odontoblasts and undifferentiated mesenchymal cells. It has recently been recognized that the

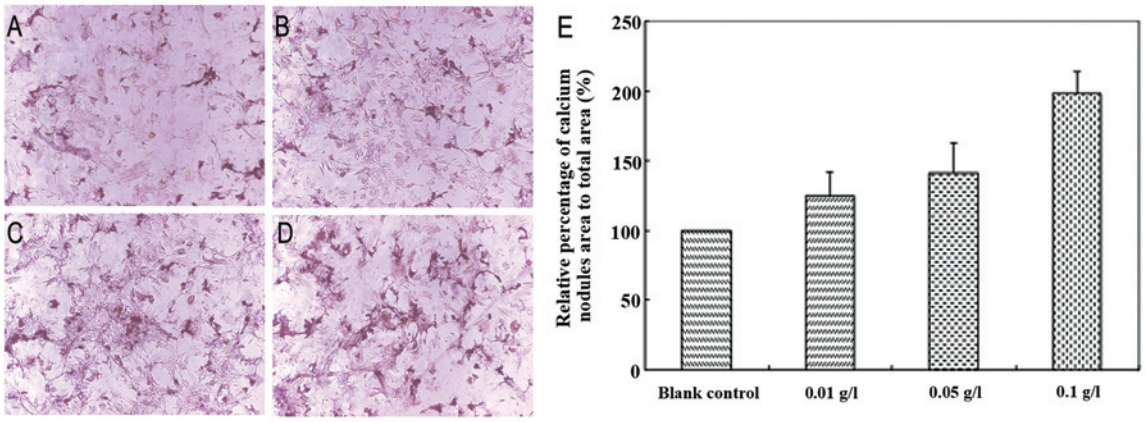

Figure 4. (A-D) Formation of calcium nodules of DPSCs after treatment with total flavonoid from *Drynaria fortunei*. (E) Relative percentage of calcium nodules area to total area after treatment of total flavonoid with *Drynaria fortunei*. DPSCs, dental pulp stem cells.

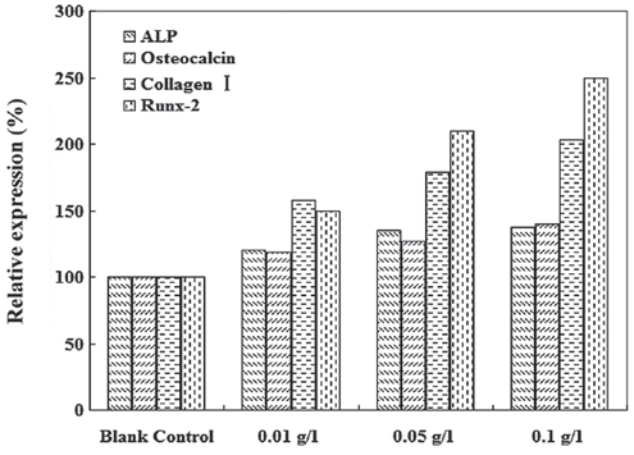

Figure 5. Osteogenic genes expression of DPSCs after treatment of total flavonoid from *Drynaria fortunei*. DPSCs, dental pulp stem cells.

undifferentiated mesenchymal cells in dental pulp tissue are DPSCs, which make up a small fraction of dental pulp tissue. The cells obtained by using explant digestion were a mixed population of dental pulp cells, we thus further isolated DPSCs from dental pulp cells by using monoplast clone culture (11). DPSCs are positive for the expression of the mesenchymal stem cell-related antigens, such as CD44, CD29, CD105 and Stro-1, but do not express stem cell markers of hematopoietic lineage such as CD34, Sca-1 and WGA (12). The presence of stem cell surface markers in rat DPSCs was examined by immunocytochemistry. In our study, DPSCs highly expressed CD44 and CD29, and scarcely expressed CD34. This result suggested that cells isolated in dental pulp cells by using explant enzyme digestion and monoplast clone culture met the criteria of DPSCs.

During bone formation, cells undergo a cascade of complex events that may include three phases: proliferation (13), osteogenic differentiation and mineralization of the extracellular matrix. Therefore, we carried out this study to investigate these three aspects.

Our findings showed that total flavonoids from *Drynaria fortunei* are capable of promoting DPSC proliferation and do not cause cytotoxicity to DPSCs. Treatment of DPSCs at concentrations of 0.01, 0.05 and 0.1 g/l total flavonoids from *Drynaria fortunei* did not change cell morphology or cell viability. CCK-8 analysis showed that the total flavonoids from *Drynaria fortunei* at concentrations of 0.01, 0.05 and 0.1 g/l for 1-5 days promoted DPSC proliferation in a dose-and time-dependent manner. Moreover, analysis of the cell cycle showed that the total flavonoids from *Drynaria fortunei* did not induce DPSC apoptosis. Thus, our results revealed that the total flavonoids from *Drynaria fortunei* at concentrations of 0.01, 0.05 and 0.1 g/l were safe to rat DPSCs, which was similar to the results reported by Hsu *et al* (14). Wang *et al* also reported that *Drynaria fortunei* has the ability to promote the proliferative activity of osteoblast-like UMR106 cells (6). To explore the mechanism of cell proliferation stimulation, we explored the effect of the total flavonoids from *Drynaria fortunei* on cell cycle distribution in DPSCs. This study showed that total flavonoid from *Drynaria fortunei* promoted DPSCs from the G0/G1 into the S phase dose-dependently. The S period is an important indication of cell proliferation, and the proportion of cells in the S period is positively associated with the ability of proliferation (15). We did not observe two typical characteristics of cells undergoing apoptosis, such as the increase of shrinking morphologies and arresting at the sub-G1 phase (16-18). This result also suggested that total flavonoids from *Drynaria fortunei* did not induce DPSC toxicity and apoptosis.

Cells in the process of osteogenic differentiation produce ALP, process procollagen to collagen, and deposit extracellular matrix-containing additional proteins (e.g., osteopontin, bone sialoprotein and osteocalsin) on the substrate, which is subsequently mineralized (19). Although the precise mechanism of ALP bone formation is poorly understood, it is widely recognized as an early biochemical marker for osteoblastic activity. The findings of our study showed that total flavonoid from *Drynaria fortunei* significantly promoted ALP activity and mRNA expression of DPSCs in a dose-dependent manner. Furthermore, the present results analyzed by the Alizarin red staining protocol revealed that the total flavonoid from *Drynaria fortunei* promoted DPSC mineralization and formation of calcium nodules.

Osteogenic differentiation induced by total flavonoid from *Drynaria fortunei* involves the regulation of ALP, osteocalcin, collagen I and Runx-2 expression. The activation of the transcription factor Runx-2 has been shown to induce the expression of ALP, osteocalcin and collagen I mRNA expression. Thus, the increase in Runx-2 expression caused by total flavonoid from *Drynaria fortunei* may engage in the regulation of ALP, osteocalcin and collagen I mRNA expression. ALP and osteocalcin are biochemical markers of the early osteogenic period, and collagen I is a marker of the middle osteogenic period (18). Our study showed that the total flavonoid from *Drynaria fortunei* is capable of enhancing DPSC osteogenic differentiation and mineralization, possibly through the Runx-2-mediated induction of ALP, osteocalcin and collagen I gene expression, and thus that one of the major reasons explaining total flavonoid from *Drynaria fortunei* promotion of osteogenic differentiation in DPSCs was its effect on regulation of expression of these osteogenic genes. The study carried out by Hung *et al* also showed that total flavonoid from *Drynaria fortunei* promoted IGF-1 expression, and then indirectly promoted the expression of various bone differentiation-related genes, such as BMP-2, BMP-6, ALP, OPN and OCN mRNA expression, but did not affect collagen collagen I mRNA expression, which is a different finding from our results (18). However, Jeong *et al* supported our results in that they found *Drynariae Rhizoma* increased the accumulation of collagen I by inhibition of the collagenase-1 expression, and also stimulate the expression of BMP-2 and ALP (7).

In conclusion, this study has shown that the total flavonoid from *Drynaria fortunei* did not affect the morphology and viability of rat DPSCs. Thus, to some extent, it is capable of promoting rat DPSC proliferation in a dose-dependent manner and the cell cycle from the G0/G1 into S phase dose-dependently. The total flavonoid from *Drynaria fortunei* has the ability to promote rat DPSCs osteogenic differentiation and mineralization. Our study further showed that the total flavonoid from *Drynaria fortunei* is capable of enhancing DPSC osteogenic differentiation and mineralization, possibly through the Runx-2-mediated induction of ALP, osteocalcin and collagen I gene expression.

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