Cibotium barometz polysaccharides stimulate chondrocyte proliferation *in vitro* by promoting G1/S cell cycle transition

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Abstract. *Cibotium barometz* polysaccharides (CBPS) are one of the most important bioactive components extracted from the *Cibotium barometz* plant, which belongs to the Dicksoniaceae family. It has been widely used for the treatment of orthopedic diseases in traditional Chinese medicine. However, the molecular mechanisms behind the therapeutic effects of CBPS remain to be clarified. In the present study, the concentration of CBPS was detected by phenol-vitriol colorimetry. Furthermore, the effects stimulated by CBPS on the viability and G1/S cell cycle transition in primary chondrocytes from Sprague-Dawley rats were investigated. A cell viability assay demonstrated that chondrocyte proliferation may be enhanced by CBPS in a dose- and time-dependent manner. The mechanism underlying the promotion of chondrocyte cell cycle was suggested to involve the stimulation of G1 to S phase transition. To further confirm the results, reverse transcription-quantitative polymerase chain reaction and western blot analyses were used to detect the expression of mRNA and protein levels of cyclin D1, cyclin-dependent kinase 4 and retinoblastoma protein. The results suggested that CBPS may stimulate chondrocyte proliferation via promoting G1/S cell cycle transition. Since osteoarthritis is characterized by deficient proliferation in chondrocytes, the present study indicates that CBPS may potentially serve as a novel method for the treatment of osteoarthritis.

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Introduction

Osteoarthritis (OA) is one of the most common joint disorders that seriously affect human health. The main pathological characteristic involves a progressive loss of articular cartilage degeneration and secondary osteophyte hyperplasia (1,2). As chondrocytes serve a crucial role in the function of the normal activity of articular cartilage (3), with deficient ability for self-repair, stimulating the functions of the chondrocytes by promotion of their proliferation may be a potential method for the treatment of OA.

Polysaccharides are a high molecular weight compound that widely exist in the cytomembrane of organisms. As demonstrated in previous studies, not only do polysaccharides enhance immunologic function and have a specific influence on tumors and viruses, but they also possess the characteristic of low toxicity (4). In recent years, numerous works on polysaccharides have been conducted and many of those have an encouraging future (5,6). However, the effect of *Cibotium barometz* polysaccharides (CBPS) on proliferation of chondrocytes cultured *in vitro* has not been investigated to date. Therefore, in the present study, an initial attempt is made in order to offer some experimental evidence for the treatment of OA using CBPS to a certain degree.

Cell proliferation is one of the most important cellular processes. The eukaryotic cell cycle is composed of 4 phases: G1, S, G2 and M. Among these stages, there are two key restriction points. The G1/S restriction point, in which DNA synthesis is prepared, is more important than the latter. The second is G2/M, in which mitosis begins. It is the G1 phase that determines whether the cell is able to continue through the cycle or withdraw. Therefore, it directly influences whether the cell continues to proliferate (7,8). Progression through each phase of the cell cycle is regulated by various cyclin-dependent kinases (CDKs) and their regulatory subunits, the cyclins (9,10). To control the cell cycle progression during the G1 phase, CDK4 and cyclin D1 bind to produce a cyclin D1-CDK4 complex that phosphorylates the retinoblastoma protein to produce phosphorylated retinoblastoma protein (pRB). pRB binds and inhibits the activity of E2F transcription factors and causes release of E2F from pRB-E2F complexes, thus the cell cycle arrest in G1 phase controlled by pRB is be

removed, so E2F transcription factors are free to bind DNA and initiate transcription required for cell cycle progression and cell proliferation (11). Therefore, Cyclin D1-CDK4-pRB complexes are able to promote cell proliferation via the promotion of cell cycle transition from the G1 to the S phase.

Cibotium barometz (CB), a type of herb used in traditional Chinese medicine, has been extensively used in Chinese medicinal formulations for the clinical treatment of orthopedic diseases such as OA (12,13). Based on previous studies, CB is composed of polysaccharides, amino acids, flavones and phenolic acids (14). In the present study, the CB polysaccharides (CBPS) were isolated. In addition, the content of polysaccharides extracted from CB is higher than in other plants from the Fujian province of China (15). The primary aim of this study was to determine the effects of CBPS on cultured chondrocytes *in vitro* via phenol-vitriol colorimetry, and the underlying mechanisms involved. It was observed that CBPS may promote chondrocyte proliferation via increasing the level of cyclin D1, CDK4 and pRB.

Materials and methods

Reagents. Fetal bovine serum (FBS) was purchased from Gibco; Thermo Fisher Scientific, Inc. (Waltham, MA, USA). Anhydrous glucose (cat. no. SG752802), sulfuric acid (cat. no. 10021618), and phenol (cat. no. 100153008) were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). MTT was purchased from Biosharp Inc. (Hefei, China), Dulbecco's Modified Eagle's medium (DMEM), trypsin-EDTA, and penicillin-streptomycin were purchased from HyClone; GE Healthcare Life Sciences (Logan, UT, USA). The type II collagenase was purchased from Sigma-Aldrich; Merck KGaA (Darmstadt, Germany) and the cell cycle detection kit was from Nanjing KeyGen Biotech Co., Ltd. (Nanjing, China), The reverse transcription system was purchased from TransGen Biotech Co., Ltd. (Beijing, China). The DNA primers were synthesized by Generay Biotech Co., Ltd. (Shanghai, China). The rabbit anti-rat cyclin D1, CDK4, and pRB antibodies were purchased from Santa Cruz Biotechnology, Inc. (cat. nos. SC-753, SC-260 and SC-32824, respectively; Dallas, TX, USA), and the anti-rabbit IgG, horseradish peroxidase-linked secondary antibody was purchased from Cell Signaling Technology, Inc. (cat. no. 7074s; Danvers, MA, USA). The study was approved by the Ethics Committee of Fujian University of Traditional Chinese Medicine (Fuzhou, China).

Extract preparation of CBPS from CB. The chosen CB was grown in Youxi, China. The dried and sliced CB sections were refluxed twice with 80% alcohol for 1 h. The solvent was dried with evaporation subsequently, and then the residue was refluxed in distilled water (100 g/l), boiled three times for 2 h and subsequently extracted. All extractions were concentrated to 100 ml under hypopiesia conditions. To conduct purification of the condensed solution, the anhydrous alcohol was used in the decoction for precipitation and incubated overnight. The precipitates isolated by centrifugation were then lyophilized. The crude polysaccharides were soaked and dissolved into distilled water (200 g/l), then the protein was removed by Sevag's method (16). The polysaccharides formed a white

power, and were dissolved in DMEM containing 10% FBS at concentration of 10 mg/ml and subsequently the solution was filtered through a 0.22 μm filter and stored at 4˚C.

Phenol-vitriol colorimetry. To prepare the glucose standard solutions, 0.1, 0.3, 0.5, 0.6, 0.7, 0.6, 0.7 ml from a stock solution (distilled water: Anhydrous glucose, 25 ml: 0.01 g) were added into 7 test tubes respectively. Each tube was supplemented with distilled water to a final volume of 1 ml, and subsequently 1 ml 5% phenol and 5 ml of 98% sulfuric acid were added for 30 min. Absorbance of distilled water, and the glucose standards was measured at 490 nm wavelength, and a standard curve of glucose concentrations based on the absorbance values was drawn. Then absorbance of CBPS was measured at 490 nm wavelength, and the concentration of CBPS was calculated based on the glucose standard curve equation.

Chondrocytes culture and identification. Male, 4-week-old Sprague-Dawley (SD) specific pathogen-free (SPF) rats (total 30) were purchased and handled in the Institutional Animal Centre affiliated to Fujian University of Traditional Chinese Medicine which was approved by the ethics committee (License no. SCXK2012-0001). Chondrocytes were isolated from the knees of rats, and were stripped and soaked with 75% ethanol for 15 min as previously described (3). The articular cartilages were cut and precisely constrained in the surface layer of cartilages ensure the purified cells were purified from the opening of the joint. Subsequently, these were transferred into PBS-containing penicillin and streptomycin and then washed three times. The cartilages were cut into a total of three 1 mm sections and digested with 0.2% type II collagenase in 50 ml culture flasks. Following this, the flasks were shaken at 37˚C. The isolated cells were collected every 2 h and the chondrocytes were cultured in 50 ml culture flasks in 4 ml DMEM containing 10% FBS at 37˚C and 5% CO_2. The culture media were changed every 2 days and the cells were subcultured at 90% confluency (17). Collagen Type II Immunohistochemical Staining was performed to confirm the identity of the chondrocytes (data not shown), as previously described from our team (3,17).

MTT assay for evaluation of cell viability. In this study, chondrocytes at passage 2 were seeded into 96-well plates at a density of $5x10^4$/ml and cultured for 24 h. They were treated with varying concentrations of CBPS (0, 100, 200, 400 and 800 μg/ml) for 24, 48 and 72 h. After stimulation, 100 μl MTT (1 mg/ml in PBS) was added to each well and incubated at 37˚C for 4 h. Subsequently, the supernatant was collected and 150 μl dimethyl sulfoxide was added to dissolve the formazan product and extracts were sonicated for 10 min. Finally, the cell viability was detected by an ELISA reader at 490 nm wave length.

Detection of cell cycle by flow cytometry. Passage 2 chondrocytes were seeded into cell culture flasks at a density of $5x10^4$/ml and cultured for 24 h. They were treated with varying concentrations of CBPS (0, 100, 200 and 400 μg/ml) for 48 h. Morphological changes of chondrocytes were observed and images were captured using an inverted phase contrast microscope (magnification, x200). The same batch of passage 2

chondrocytes seeded in 6-well plates at a density of 1×10^4/ml were subsequently subjected to DMEM without FBS for 24 h to synchronize the cell cycle stage. Various concentrations of CBPS (0, 100, 200 and 400 μg/ml) were added into the plates for 48 h, then the cells were collected and the cell density was adjusted to 1×10^5/ml. To evaluate the phase of chondrocyte proliferation, three solutions (A, B and C) were incubated using the cell cycle detection kit (Nanjing KeyGen Biotech Co., Ltd.) according to the manufacturer's instructions. The results were analyzed by ModFit software version 3.0 (Verity Software House, Inc., Topsham, ME, USA). Therefore, cells in each phases of the cell cycle (G0/G1, S and G2/M) could be detected using a flow cytometer.

RNA extraction and reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. The chondrocytes were treated with the different concentrations of CBPS (0, 100, 200 and 400 μg/ml) for 48 h. The total RNA was extracted from which in accordance with the standard instructions of the TRIZOL reagent (Thermo Fisher Scientific, Inc.). For RNA samples with 260/280 absorbance between 1.8 and 2.0, 1 μg RNA was reverse transcribed into cDNA based on the instructions provided by TransGen Biotech Co., Ltd. (cat. no. H41220K060; Beijing, China). The PCR reactions were prepared, heated to 90°C for 5 min and subjected to 35 cycles of denaturation at 94°C for 30 sec, annealing at the specific Tm for 30 sec, and extension at 72°C for 45 sec to complete amplification. The expressions of cyclin D1, CDK4, RB and β-actin mRNA were analyzed by PCR. β-actin was used as an internal control. The primer sequences provided by the manufacturer were as follows: Cyclin D1 (product size, 399 bp), sense 5'-AGC AGA AGT GCG AAG AGG AGG TC-3' and antisense 5'-GGA AAG AAA GTG CGT TGT GCG GTA G-3'; CDK4 (product size, 494 bp), sense 5'-CCT ACG GAC ATA CCT GGA CAA-3' and antisense 5'-GAG GCA ATG AGA TCA A-3'; RB (product size, 255 bp), sense 5'-CTT TAT TGG CCT GTG CTC TTG-3' and antisense 5'-ATT CCA TGA TTC GAT GCT CAC-3'; β-actin (product size, 385 bp), sense 5'-GGG AAG TGC TGG ATA G-3' and β-actin, antisense 5'-GTG ATG TTT CGG ATG G-3'. The DNA bands were detected by gel electrophoresis (1.5% agarose) and analyzed by the Gel Documentation system (Gel Doc 2000; Bio-Rad Laboratories, Inc., Hercules, CA, USA). The experiment was performed 3 independent times.

Western blot analysis. Following treatment of chondrocytes with different concentrations of CBPS (0, 100, 200 and 400 μg/ml) for 48 h, the total protein was extracted from the cells by radioimmunoprecipitation assay (RIPA) lysis buffer supplemented with 1 mM phenylmethanesulfonyl fluoride (PMSF), according to the manufacturer's instructions (Beyotime Institute of Biotechnology, Haimen, China). Subsequently the protein concentrations were determined by a bicinchoninic acid assay kit and a standard curve was produced. Total proteins (25 μg/lane) were separated by electrophoresis on 12% SDS-PAGE gels and transferred onto polyvinylidene membranes correspondingly. Then the membranes were blocked for 2 h in 5% skimmed milk at room temperature. Following blocking, the membranes were washed in TBS-Tween for 5 min and then incubated with the primary
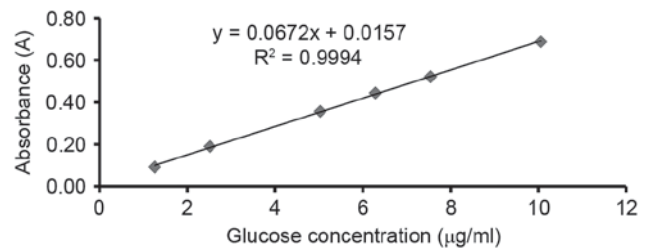

Figure 1. Standard curve for detecting the content of the CBPS by phenol-sulfuric acid colorimetry. CBPS, *Cibotium barometz* polysaccharides.

antibody for cyclin D1, CDK4, pRB and β-actin (1:1,000) at 4°C overnight. Following a second wash using TBS-Tween, the membranes were incubated with the secondary antibody solution (1:5,000) for 1 h at room temperature. Three final 10 min washes were performed using TBS-Tween. Finally, the antibody-bound protein bands were examined with enhanced chemiluminescence (Beyotime Institute of Biotechnology) and the results of images were captured from 3 independent experiments using a Kodak image station 400R (Kodak, Rochester, NY, USA).

Statistical analysis. The results are expressed as the mean ± standard deviation. Statistical analysis was performed by Student's t-test or analysis of variance followed by Bonferroni post hoc test, using SPSS software (version 18.0; SPSS Inc., Chicago, IL, USA). $P < 0.05$ was considered to indicate a statistically significant difference.

Results

The concentration of CBPS detected by phenol-vitriol colorimetry. There was a favorable linearity relationship between the absorption and content determined in the range of 1-11 μg/ml, and the regression equation $y = 0.0672x + 0.0157$, $R^2 = 0.9994$ (Fig. 1). The content of polysaccharides was ~76.91% with good precision of relative standard deviation (RSD) at 1.8,605% and well stability of RSD at 1.9,883%, the result of the average recovery test was 95.4% and RSD of 1.83%. These results identified that the CBPS was at an acceptable level.

Morphological characteristics for chondrocytes. The primary chondrocytes cultured *in vitro* were observed by phase microscopy (x200 magnification). From the morphological images (Fig. 2), the different changes of the chondrocytes over time in culture were observed and the further descriptions have been presented in a previous study (18). Following observation, passage 2 chondrocytes were regarded as optimal for their abundant extracellular matrix and convenient cultured conditions in the present study. The passage 2 chondrocytes had a characteristic cobblestone-like morphology and moderate counts, with clear boundaries and distinct nuclei.

Chondrocyte proliferation was promoted via CBPS. An MTT assay was used to detect the cell proliferation following culture with different concentrations of CBPS (0, 100, 200,
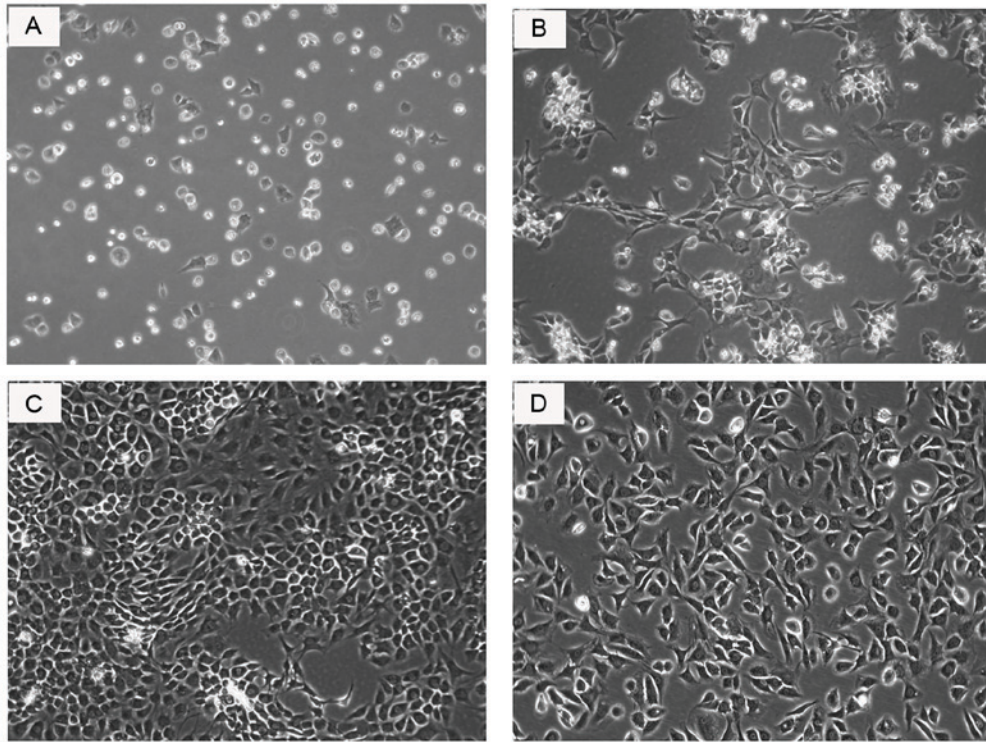
Figure 2. Morphological changes of chondrocytes were observed and images were collected by inverted phase contrast microscope (magnification, x200). Images of primary cells cultured for (A) 24 h, (B) 4 days, (C) and 8 days, and (D) chondrocytes at passage 2.
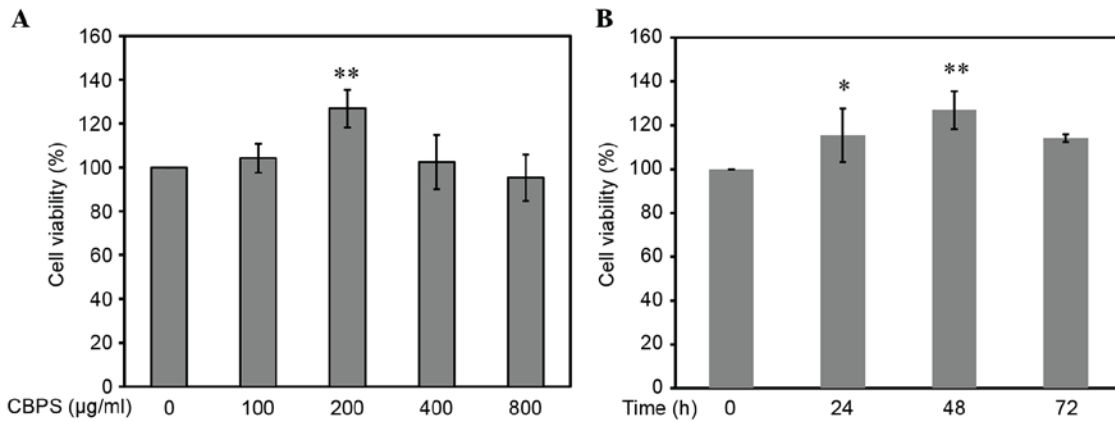

Figure 3. Effect of CBPS on the proliferation of chondrocytes. MTT assay was used to detect the chondrocyte proliferation following treatment with different concentrations of the CBPS (0, 100, 200, 400 and 800 μg/ml) for (A) 48 h or at (B) 200 μg/ml (0, 24, 48 and 72 h). The data are presented as mean ± standard deviation. *P<0.05, **P<0.01, vs. the control group (0 μg/ml). CBPS, *Cibotium barometz* polysaccharides.
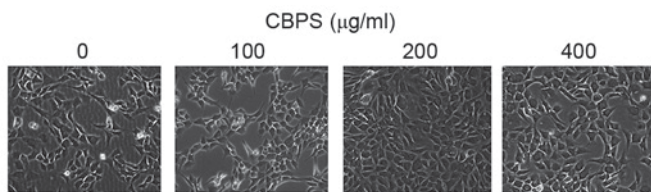

Figure 4. Morphological changes of the chondrocytes following CBPS treatment at 0, 100, 200 and 400 μg/ml for 48 h (magnification, x200). CBPS, *Cibotium barometz* polysaccharides.

400 and 800 μg/ml) at 24, 48 and 72 h. As presented in Fig. 3A, at 48 h, the optical density (OD) of the 200 μg/ml CBPS group was significantly higher compared with the 0 μg/ml group (P<0.01). When comparing the 100, 400 and 800 μg/ml groups, there were no significant differences in the viability of the chondrocytes compared with the 0 μg/ml group (P>0.05). Fig. 3B illustrates the viability of chondrocytes treated with 200 μg/ml CBPS for 24, 48 and 72 h. Chondrocyte viability significantly increased at 24 h of treatment, and was further increased at 48 h, compared with the 0 h group (P<0.05 and P<0.01 respectively; Fig. 3B). These findings suggest that chondrocyte proliferation is likely promoted by CBPS treatment in a time-dependent manner. The optimal time and concentration of CBPS treatment was 48 h and 200 μg/ml, respectively (Fig. 3).
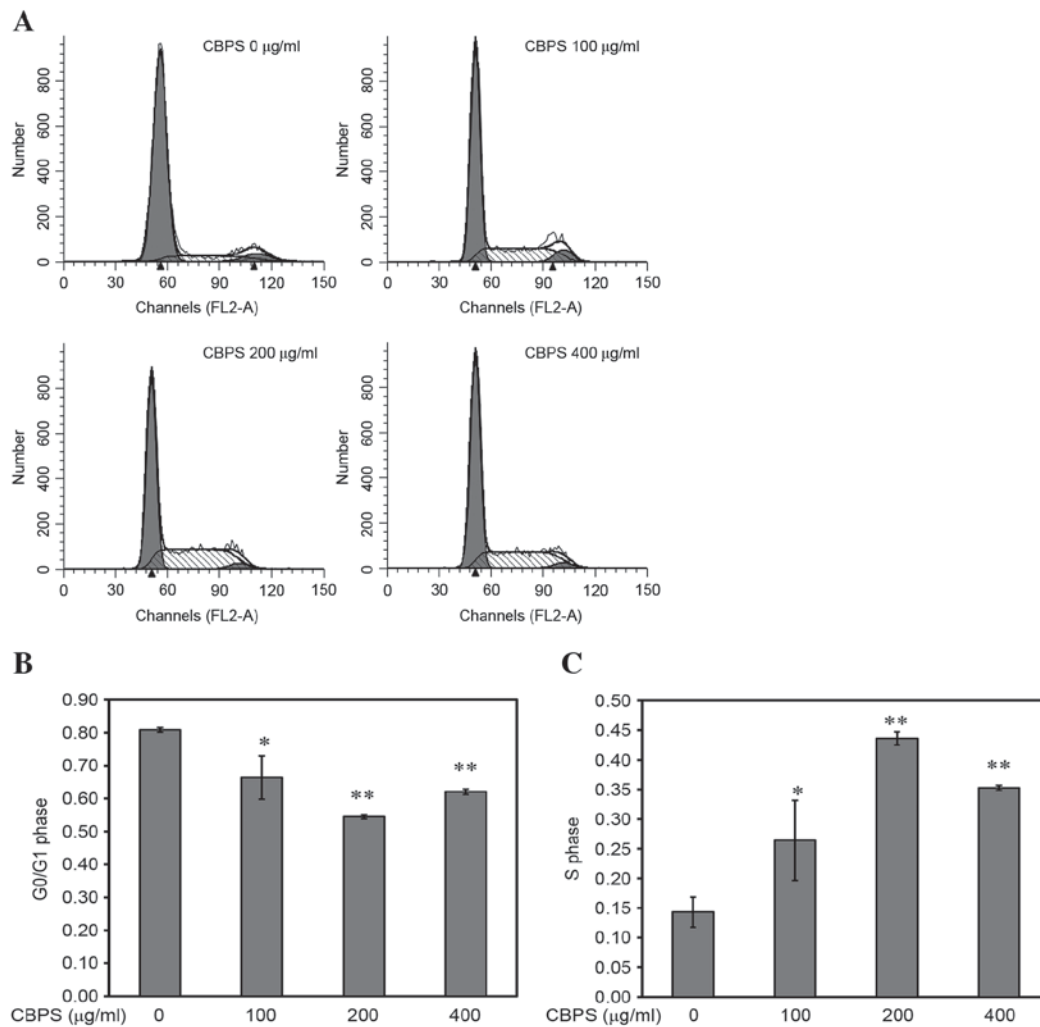
Figure 5. Flow cytometry results following CBPS treatment. (A) Cell cycle changes of chondrocytes. (B) Proportion of chondrocytes in the G0/G1 phase following CBPS treatment. (C) Proportion of chondrocytes in the S phase following CBPS treatment. The data are presented as mean ± standard deviation, [*]P<0.05, [**]P<0.01 vs. the control group (0 μg/ml). CBPS, *Cibotium barometz* polysaccharides.

Effects of CBPS on the chondrocyte cell cycle. Based on the aforementioned results, the range of intervening concentrations was reduced to 0, 100, 200 and 400 μg/ml. Following treatment of chondrocyte with these concentrations for 48 h the morphology of chondrocytes was observed (Fig. 4). Flow cytometry analysis identified the effects of CBPS (0, 100, 200 and 400 μg/ml) on the chondrocyte cell cycle (Fig. 5A). The proportion of cells in the G0/G1 phase (Fig. 5B) was lowest in the 200 μg/ml CBPS-treated group (0 μg/ml, 0.8088±0.0069; 100 μg/ml, 0.664±0.0659; 200 μg/ml, 0.5451±0.0065; and 400 μg/ml, 0.6209±0.0083). Compared with the 0 μg/ml group, CBPS treatment caused significant decreases in proportion of G0/G1 cells (100 μg/ml, P<0.05; 200 μg/ml, P<0.01; and 400 μg/ml, P<0.01). The percentage of cells in S phase (Fig. 5C) in the 200 μg/ml group was significantly higher compared with all other CBPS treatment groups (0 μg/ml, 0.1434±0.0254; 100 μg/ml, 0.2642±0.067; 200 μg/ml, 0.4362±0.0110; and 400 μg/ml, 0.3530±0.0041). When compared with the 0 μg/ml group, there were significant increases in the proportion of cells in S phase following CBPS treatment (100 μg/ml, P<0.05; 200 μg/ml, P<0.01; 400 μg/ml, P<0.01). When comparing with the 100 and 400 μg/ml group, there were no significant differences in the G0/G1 and S phase proportions (P>0.05). This suggested an opposite trend to the G0/G1 phase. Therefore, the proliferation of chondrocytes could be promoted by CBPS in the progression of the cell cycle in the transition from G1 to S phase.

CBPS upregulates the expression of cyclin D1, CDK4 and RB mRNA and protein. It is necessary to examine for the mechanism underlying CBPS-induced promotion of chondrocyte proliferation. Therefore, the mRNA and protein expression levels of cyclin D1, CDK4 and RB were tested by RT-qPCR and western blot analysis, respectively. As presented in Fig. 6, the mRNA expression of cyclin D1, CDK4 and RB in CBPS-treated groups was significantly higher compared with the 0 μg/ml group, with peak mRNA expression levels in the 200 μg/ml group (P<0.01). When measuring the protein expression levels by western blotting, cyclin D1, CDK4 and pRB were again significantly upregulated in the CBPS-treated groups compared with the 0 μg/ml group, with peak protein expression levels in the 200 μg/ml group (P<0.01; Fig. 7).
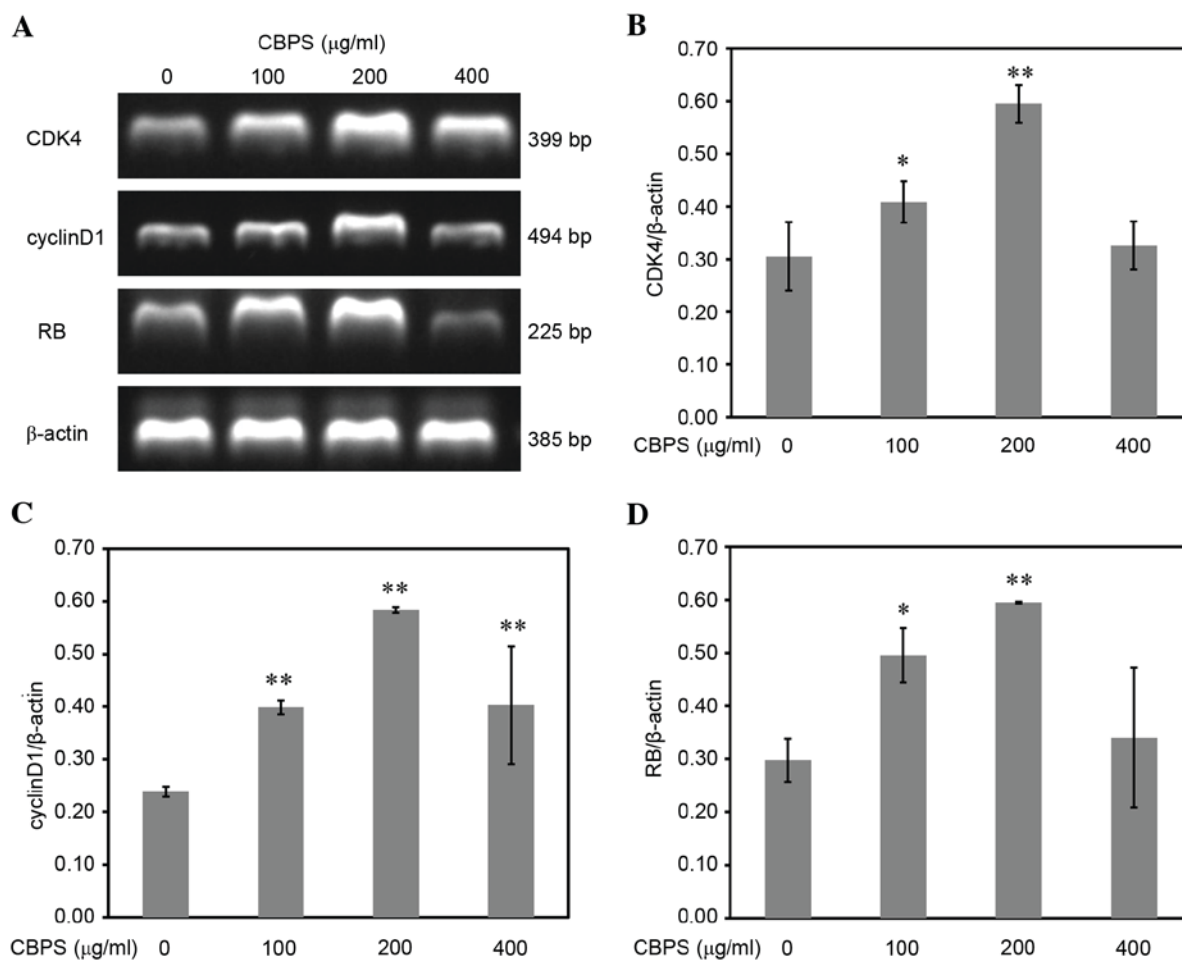
Figure 6. mRNA expression of CDK4, cyclin D1 and RB determined using reverse transcription-polymerase chain reaction. (A) Expression of CDK4, cyclin D1, RB and β-actin mRNA in the chondrocytes following CBPS treatment. (B) Relative mRNA expression of CDK4. (C) Relative mRNA expression of cyclin D1. (D) Relative mRNA expression of RB. The data are presented as mean ± standard deviation. $^*P<0.05$, $^{**}P<0.01$, vs. the control group (0 μg/ml). CDK4, cyclin-dependent kinase 4; RB, retinoblastoma; CBPS, *Cibotium barometz* polysaccharides.

Discussion

The incidence of osteoarthritis (OA) still poses a major public health challenge throughout the world, and, currently there is no thoroughly effective therapy available. Considering that chondrocytes are responsible for secreting extracellular matrix, and the maintenance of cartilage homeostasis is involved in the pathological characteristics of OA, promoting chondrocyte proliferation may be a prospective and potential method for the treatment of OA (19-21).

Polysaccharides are biological molecules that can store bioenergy and support the role of structural components (22), which are necessary for the entire organism. In recent years, polysaccharides have also been demonstrated to be important in protecting various physiological functions (23,24). Polysaccharides are widely extracted from Chinese herbs. In the present study, the CBPS were obtained from one of the most important Chinese traditional herbs used in OA treatment, *Cibotium barometz*. In order to explore the mechanisms behind the effects of CBPS on chondrocytic functions, chondrocytes were treated with varying concentrations of CBPS (100, 200, 400 and 800 μg/ml) and with 200 μg/ml CBPS for 24, 48 and 72 h. An MTT assay was then used to test the effect of CBPS on the total numbers of viable chondrocytes.

According to the results from the MTT assay, the proliferation of chondrocytes is promoted by CBPS in a time-dependent manner, and the optimum time and concentration were 48 h and 200 μg/ml, respectively.

With regards to the transmission in the cell cycle, cyclins and CDKs are positive regulators responsible for proliferation; however, CDK inhibitors (CDKI) are negative regulators that control the speed of the cell cycle. The balance of negative and positive regulation are key factors that determine the changes of the various phases in the cell cycle (25,26). The cell cycle is formed of 4 stages: G1, the preparation for DNA synthesis; S, DNA synthesis; G2, the preparation for mitosis; and M, mitosis. Among the four stages, the S and M phases are the two most crucial processes.

The G1/S and G2/M transitions are vital steps of cell cycle progression. To further investigate the cell cycle in chondrocytes, the cells were treated with 0, 100, 200 and 400 μg/ml CBPS for 48 h. Subsequently, flow cytometry was used to detect cells in the different stages of the cell cycle. In the present study, the results demonstrated that the percentage of chondrocytes in the G0/G1 phase was obviously reduced and the percentage of chondrocytes in the S phase was evidently increased by CBPS, demonstrating that chondrocyte proliferation may be stimulated by CBPS treatment via promotion of the transition
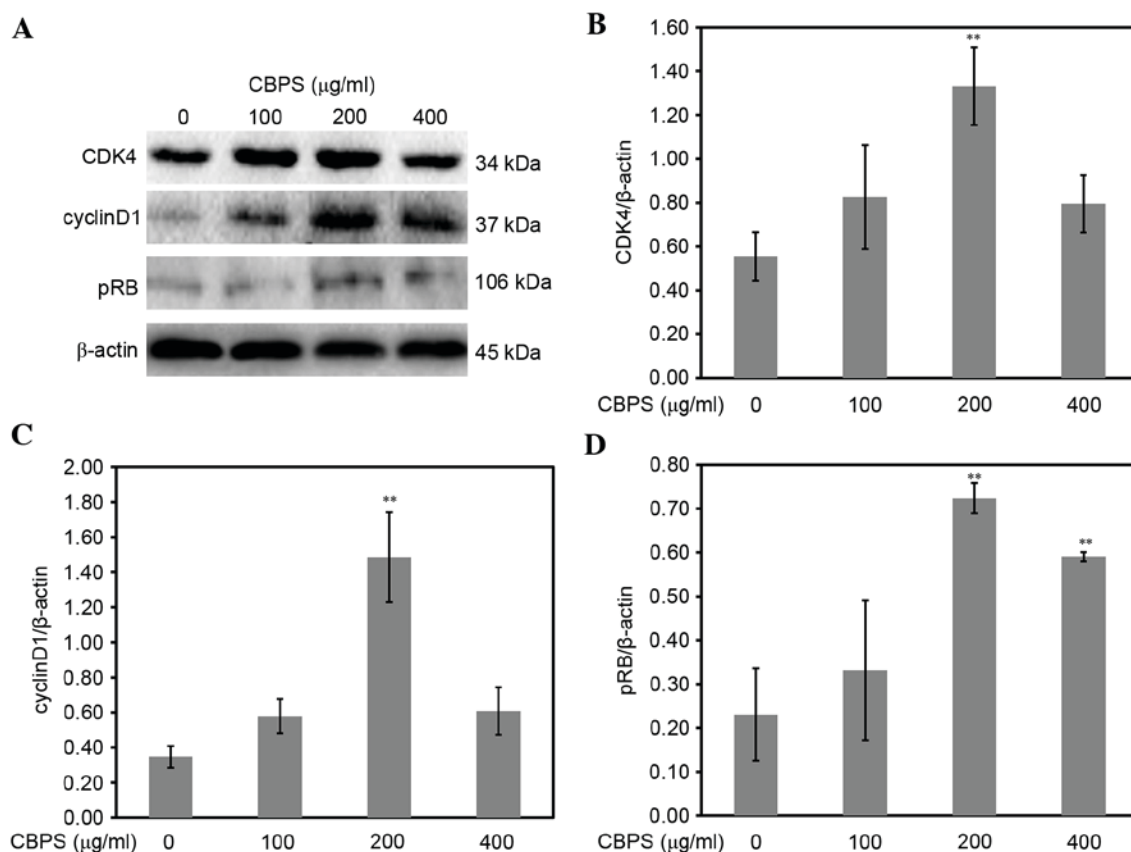
Figure 7. Protein expression of CDK4, cyclin D1 and pRB using western blot analysis. (A) The expression of CDK4, cyclinD1, pRB and β-actin protein in the chondrocytes via CBPS. (B) Relative protein expression levels of CDK4. (C) Relative protein expression levels of cyclin D1. (D) Relative protein expression levels of pRB. The data are presented as mean ± standard deviation. *P<0.05, **P<0.01, vs. the control group (0 μg/ml). CDK4, cyclin-dependent kinase 4; pRB, retinoblastoma protein; CBPS, *Cibotium barometz* polysaccharides.

from G1 to S phase. Cyclin D1-CDK4-pRB complexes are composed of cyclins, CDKs and pRB, which are basic protein families that regulate the progression of the cell cycle. Once cyclin D1-CDK4 complexes phosphorylate pRB, which releases pRB from interaction with E2F transcription factors allowing them to bind DNA and trigger transcription to directly regulate the progression from G1 phase to S phase, consequently promoting chondrocyte proliferation and restraining chondrocyte apoptosis (27,28).

Based on the results in the current study, which demonstrated that CBPS treatment may boost the mRNA and protein expression of cyclin D1, CDK4 and pRB, indicating that CBPS promotes the progression of chondrocytes from the G1 to the S phase by regulating the cyclin D1-CDK4-pRB complex. In conclusion, these data indicated that CBPS may effectively increase chondrocyte proliferation by promoting G1/S cell cycle transition and enhance the mRNA and protein expression of cyclin D1, CDK4 and pRB.

However, as one of the most misunderstood and complex diseases, the exact pathological and pathogenesis characteristics of OA still have not been clarified in full. Based on a recent meta-analysis of the curative effect of dextrose prolotherapy for knee osteoarthritis, dextrose prolotherapy may improve pain and physical function in patients with OA (29). Previous research has validated the safety of dextrose prolotherapy for clinical treatment of OA (30,31), and this suggested that future studies should investigate the connection between the CBPS and OA. The mechanism underlying the effects of CBPS on OA should be explored further. Alongside the current advances in scientific research, the biologically active ingredient of CBPS should be purified and synthesized, in order to determine its curative effects in relevant clinical trials.

Acknowledgements

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