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Procyanidin B2 ameliorates the progression of osteoarthritis: An in vitro and in vivo study

Wenxiang Cai, Yubiao Zhang, Wenyi Jin, Sixing Wei, Junwen Chen, Changheng Zhong, Yujian Zhong, Chang Tu*, Hao Peng

Department of Orthopedics, Renmin Hospital of Wuhan University, Wuhan 430060, Hubei Province, PR China

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ABSTRACT

Objective: Osteoarthritis (OA) is characterized by cartilage degeneration and inflammation. Procyanidin B2 (PCB2), a natural flavonoid compound, exhibits potential anti-inflammatory and anti-oxidative effects against several diseases. However, its curative effects on OA remain unclear.

Purpose: Herein, we explored the anti-arthritic effects of PCB2 on OA onset and progress and its potential mechanism.

Methods: CCK-8 assays and EdU staining were used to assess the cytotoxic effects and cell proliferation activity of PCB2. Flow cytometry was used to detect apoptosis in chondrocytes. ELISA, qPCR, and western blotting, were applied to explore the expression of apoptosis and senescence-associated secretion phenotype (SASP) factors. The Nrf2/NF-κB signaling cascade was explored using immunofluorescence and western blotting. Additionally, we silenced the Nrf2 gene using siRNAs to verify its function in PCB2 regulation of senescence and apoptosis phenotypes. Safranin O-Fast Green (SO) and immunohistochemical staining were used to explore the effects of PCB2 on OA model rats.

Results: PCB2 dampened interleukin (IL)-1β-triggered expression of SASP factors in vitro. Additionally, PCB2 diminished IL-1β-triggered destruction of the extracellular matrix (ECM) via downregulating the expression of MMPs, while upregulating the expression of collagen II and aggregan. In addition, PCB2 treatment reduced IL-1β-induced apoptosis of chondrocytes. Mechanistically, PCB2 could attenuated chondrocyte senescence in vitro via the Nrf2/NF-κB pathway. Moreover, PCB2 exhibited anti-apoptotic properties via the Nrf2/BAX/Bcl-2 pathway. PCB2 alleviated knee cartilage degeneration in an OA rat model.

Conclusions: Our results suggest that PCB2 may be used as a therapeutic agent for OA.

1. Introduction

Osteoarthritis (OA) is a chronic arthropathy that mostly affects the elderly, and its prevalence increases with age [1–3]. Patients with OA present with disrupted subchondral bone, articular cartilage, and synovial tissue, leading to pain, joint dysfunction, and tissue loss [4,5]. In addition to the high pain intensity and disability associated with OA, the burden of care and treatment costs for the disease was estimated to reach $27 billion annually in 2016 in the United States of America [6]. OA can induce joint discomfort and abnormalities as well as restrict normal function, all of which reduce the quality of life [7]. Despite the discovery of risk factors, including obesity, trauma, and inheritance, the onset of OA remains elusive [8,9]. Furthermore, owing to the fact that existing pharmacological therapies can only relieve clinical symptoms momentarily, finding novel agents that can reverse the course of OA with fewer adverse effects is of great importance [5,10].

Chondrocytes, the only cell type in articulation, are important modulators of the production of extracellular matrix (ECM), such as proteoglycans and collagen, which are necessary for cartilage structure and function [11]. In OA, chondrocyte apoptosis and senescence have been observed and are linked to chondrocyte destruction and loss of ECM [12,13]. Interleukin (IL-1) is implicated in the pathogenesis of OA...
IL-1β causes OA by inducing apoptosis and senescence in chondrocytes [15]. ECM deterioration is caused by increased chondrocyte apoptosis and senescence, which leads to the development of OA. Thus, research on strategies for preventing chondrocyte senescence and aberrant apoptosis caused by inflammation will aid in a better comprehension of cartilage deterioration, and will be of practical utility in the treatment of the disease.

Cellular senescence is a long-term condition of cell-cycle arrest that may be produced by a variety of factors and is linked to age-related disorders [6,16]. Senescence is marked by increased p16 (INK4A) and p21 (CIP) protein expression, which may be identified using the senescence-associated β-galactosidase (SA-β-gal) assays [6]. According to recent research, senescent cells may not only have a passive phenotype, but may actively harm nearby cells [6]. The senescence-associated secretory phenotype (SASP) refers to the ability of senescent cells to produce multiple types of inflammation-promoting factors such as growth factors, chemokines, cytokines, and proteases [17,18]. SASP factors may affect the microenvironment in tissues, leading to local or systemic dysfunction, and may induce senescence of surrounding cells by paracrine factors [19,20]. Senescence is a primary risk factor for many chronic degenerative diseases, including OA [17,21]. OA could be alleviated by systemic removal of p16-positive senescent cells [17], which could repress the cell cycle as well as SASP, cell senescence, and oxidative stress [22]. Taken together, these studies suggest that chondrocyte senescence is a causative factor for OA.

Procyanthoinidins, the most abundant flavonoid compounds in grape seeds. One of the most frequently found procyanthoinidins is procyanidin B2 (PCB2), which is comprised of two molecules of flavan-3-ol (-)-epicatechin [23]. PCB2 alleviates oxidative stress in liver cells by activating the Nrf-2/Keap1 signaling cascade and repressing NF-xB signalling [23]. Additionally, PCB2 protects intestinal stem cells by slowing down the degradation of Nrf2 and activating Nrf2 in the nucleus, which leads to subsequent antioxidant enzyme expression [24]. Furthermore, PCB2 suppresses the levels of expression of senescence markers [25]. However, its role in the prevention of OA remains unclear. Hence, further research is necessary to explore whether PCB2 protects chondrocytes and prevents OA progression. Here, the impact of PCB2 on IL-1β-induced rat articular chondrocytes and the underlying molecular mechanisms were explored. We also explored whether PCB2 could prevent OA development.

2. Materials and methods

2.1. Reagents

PCB2 (C30H26O12, purity ≥ 99 %) was purchased from MedChemExpress (USA). Recombinant rat IL-1β (P6245) was supplied by Beyotime (Shanghai, China). ELISA kits for IL-6 (CSB-E04640r) and MMP10 (CSB-E07414r) were acquired from CUSABIO (Wuhan, China). ELISA kits for IL-6 (CSB-E04640r) and MMP10 (CSB-E07414r) were purchased from Servicebio (ABclonal, USA). The primary antibodies used were anti-MMP3 (A11418, ABclonal), anti-MMP10 (CSB-E07414r), anti-GAPDH (10494-1-AP, ABclonal), anti-IL-6 (21865-1-AP, ProBeyotime (Shanghai, China). ELISA kits for IL-6 (CSB-E04640r) and MMP10 (CSB-E07414r) were purchased from Servicebio (ABclonal, USA). The primary antibodies used were anti-MMP3 (A11418, ABclonal), anti-MMP10 (A3033, ABclonal), anti-lamin B (12987-1-AP, Proteintech), anti-GAPDH (10494-1-AP, ABclonal), anti-IL-6 (21865-1-AP, Proteintech), anti-IL-8 (A2541, ABclonal), anti-p16 (10883-1-AP, Proteintech), anti-p21 (A1843, ABclonal), anti-p-p65 (ab76302, Abcam), anti-p-p65 (66535-1-lg, ABclonal), anti-IL-1β (AP0707, ABclonal), anti-Nrf2 (A0674, ABclonal), anti-HO-1 (A19693, ABclonal), anti-Bax (19684, ABclonal), anti-caspase-3 (A2156, ABclonal), anti-β-galactosidase (A12045), and anti-collagen II (A1560, ABclonal). The secondary HRP-linked antibodies were used as anti-rabbit IgG (GR23302) and FITC-labeled goat anti-rat IgG, which were purchased from Servicebio (ABclonal, USA).

2.2. Isolation, culture, and identification of cells

All animal studies were authorized by the Wuhan University Committee on the Use and Care of Animals. The 1-day-old Sprague-Dawley rats were sacrificed at the short neck, and the cartilage tissue was separated from the joint, cut into tissue blocks smaller than 1 mm³, and washed twice with PBS containing double antibodies. First, 0.25 % trypsin, three times the volume, was placed in a 37°C constant temperature shaker for 15–20 min. After discarding the supernatant, 0.2 % collagenase II was added, and the mixture was placed in a 37°C constant temperature shaker for 4–6 h. The cells were suspended in a high glucose DMEM/F12 medium and cultured in a 5 % CO₂ incubator at 37°C. In our study, we used 1–3 passages of chondrocytes.

2.3. Cell viability assay

The CCK8 assay was utilized to evaluate chondrocyte viability. Briefly, chondrocytes were seeded evenly in a 96-well plate at a density of 5000 cells/well. The cells were treated with PCB2 at different concentrations (0, 5, 10, 20, 40, and 80 µM) for 24 h. In addition, cells were exposed to PBC2 at various concentrations (0, 5, 10, 20, and 40 µM) for 24 h and then stimulated with IL-1β (10 ng ml⁻¹) for 12 h. After treatment, 100 µL of DMEM/F12 medium containing 10 µL CCK8 solution was added to each well, the plate was incubated for 2 h, and the absorbance was determined at 450 nm using an enzyme-labeling instrument (Tecan Sunrise, Salzburg, Austria).

2.4. Senescence-associated β-galactosidase staining

After treated with PCB2 and IL-1β, the chondrocytes were cultured overnight in the dark at 37°C with mixed staining liquid. Cells were observed under a light microscope (Nikon, Japan).

2.5. Measurement of cell apoptosis

The chondrocytes were treated as described, the culture medium was transferred to a clean centrifuge tube, and the cells in the culture bottle were washed with PBS. The supernatant was discarded, and the cells were collected. A total of 1×10⁵ suspended cells was collected and centrifuged at 300×g for 5 min. Annexin V-binding buffer diluted to 1×500 µL was added to resuspend the cells. Annexin V (5 µL) and nuclear staining solution (5 µL) were added to the cell suspension. After mixing gently and vigorously, the suspension was incubated for 15–20 min at room temperature. Finally, flow cytometry was performed.

2.6. RT-PCR

Total RNA was extracted from the cells using KIT. The concentration and purity of RNA were determined using a specific instrument. The Hiscrypt II QT Supermix was used for qPCR (SYBR Green), and the sample containing 2 mg total RNA was reverse transcribed into cDNA. Quantitative reverse transcription was repeated three times on a CFX96 real-time PCR system. Amplification was carried out under the following conditions: 95°C for 3 min, 95°C 10 s, 60°C 30 s, and 95°C 15 s for 49 cycles. The expression of MMP3, MMP10, IL-6, and IL-8 was analyzed using RT-PCR, and the 2⁻ΔΔCt method was used for calculation of relative levels (see Table 1).

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer (5' → 3')</th>
<th>Reverse primer (5' → 3')</th>
</tr>
</thead>
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<tr>
<td>MMP3</td>
<td>CGGTTCGCGCTGTCGTTCAAG</td>
<td>GCGCAAAAGTGCTGTCCTT</td>
</tr>
<tr>
<td>MMP10</td>
<td>TGCTCTGCTACCTTCCTGTAG</td>
<td>TCACATCTTCCTTGAGTTGTA</td>
</tr>
<tr>
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<tr>
<td>P16</td>
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</tr>
<tr>
<td>COL1I</td>
<td>TGGACGGTCATGAGGTTTCTCT</td>
<td>TGAGGACGGATTGCTACTCCT</td>
</tr>
<tr>
<td>GAPDH</td>
<td>GGAACCTGACCTGCGCTCTAG</td>
<td>GTAGCCACGATGGCCTGTA</td>
</tr>
</tbody>
</table>
2.7. Western blotting

After the chondrocytes were inoculated with PCB2 and IL-1β, total intracellular protein was extracted by dissolving the chondrocytes in RIPA buffer containing phosphatase and protease inhibitors on ice for 40 min. After electrophoresis and electrotransfer, the membranes were incubated with primary antibodies against MMP3, MMP10, lamin B, GAPDH, IL-6, IL-8, p16, p-p65, p65, Nrf2, HO-1, IkBα, Bcl-2, caspase-3, Bax, aggrecan, and collagen II overnight at 4 °C. Next, the membranes were incubated with HRP secondary antibody at 37 °C for 1 h. Each group of data was tested at least three times. Finally, the level of the protein bands was visualized using Image J software (version 3.0).

2.8. Immunofluorescence staining

After experimental treatment, the cells were incubated in 4 % paraformaldehyde for 10 min at room temperature for fixation, permeabilized with 0.25 % Triton X-100 PBS solution, and blocked with PBS supplemented with 0.1 % Tween 20 and 1 % BSA at 37 °C for 30 min. Subsequently, primary antibody (anti-NF-κB P65, anti-collagen II, and anti-Nrf2; 1:200) was added and the mixture incubated overnight at 4 °C. Next, the cells were incubated with fluorescent secondary antibody at 37°C for 1 h and stained with DAP for 10 min. After washing three times, the samples were sealed with anti-fluorescence quenching tablets, and photographs were taken under a fluorescence microscope (Nikon, Tokyo, Japan).

2.9. siRNA transfections

Target siRNA against Nrf2 (si-Nrf2) and control siRNA were acquired from RiboBio (Guangzhou, China). Chondrocytes were transfected with Nrf2 siRNA and negative control (NC) siRNA using RNAiMAX Reagent according to the manufacturer’s instructions.

2.10. Molecular docking

The model of PCB2 was acquired from the PubChem database (http://pubchem.ncbi.nlm.nih.gov/). The three-dimensional structure of PCB2 was produced by Discovery Studio 2016. Nrf2 (PDB ID: 2LZ1) was acquired from RCSB Protein Data Bank (https://www.rcsb.org/). Then the Nrf2 molecule was inputted into PyMOL software and treated by the procedures of cleaning, preparing, removing water and adding hydrogen. Finally, the results of molecular docking were evaluated by binding affinity values.

2.11. Animal OA model

All surgical interventions and treatment procedures were authorized by the Animal Ethics Committee of Wuhan University. Thirty male Sprague–Dawley rats (200–250 g, 10-week old) with uniform body weights were maintained in a specific pathogen-free environment at suitable temperature (22–26 °C), humidity (50 %–70 %), and illumination (12-h-light/12-h-dark cycle). According to the previous literature [26], Destabilization of the medial meniscus (DMM) is the standard method for establishing experimental OA. Briefly, the rats were anesthetized with isoflurane, the medial joint capsule of the right knee was dissected using microsurgical scissors, and the medial meniscus-tibial ligament was cut. After surgery, the rats were divided into three groups (n = 10): sham, DMM, and DMM + PCB2 groups. PCB2 (40 μg kg⁻¹) was orally administered to the DMM + PCB2 group twice a week postoperatively. Rats in the DMM and sham groups were treated with saline twice a week, postoperatively. After 8 weeks, the rats were sacrificed to collect knee joint tissues. Tissues were fixed in 4 % paraformaldehyde for subsequent analyses.

2.12. Histopathological analysis and immunohistochemical staining

The collected joint tissues were decalcified, dehydrated, embedded in paraffin, and cut into 5 μm sections and stained with Safranin O-Fast Green (S–O). The Osteoarthritis Research Society International (OARSI) scoring system was used for histological assessment [27]. Cartilage degeneration was evaluated using a microscope (Olympus, Japan) and graded using a histological grading system.

After dewaxing, hydration, antigenic repair, and sealing with H₂O₂, the tissue sections were blocked with 1 % goat serum at 37 °C for 1 h, followed by overnight incubation at 4 °C with appropriate concentrations of primary antibody (anti-collagen II, anti-p16 (INK4A), anti-p21, anti-MMP13, anti-Bax, anti-p-p65, anti-Nrf2, and anti-Bcl2; 1:200). Next, HRP secondary antibody was incubated at 37 °C for 1 h. DAB staining was completed and tissue sections were photographed for further analysis. Each group of data was tested at least three times.

2.13. Statistical analysis

The data are presented as the mean ± SD. The data were analyzed using SPSS or GraphPad Prism 20.0 software, and ANOVA followed by Tukey’s test for comparison between the control and intervention groups. Statistical significance was set at P < 0.05. Each set of data was analyzed at least three times.

3. Results

3.1. Effects of PCB2 on the viability of chondrocytes

The structure of PCB2 is shown in Fig. 1A. Chondrocyte identification is shown in Fig. 1B. PCB2 exerted no significant cytotoxic effects at concentrations of 0, 5, 10, 20, 40, and 80 μM (Fig. 1C). Therefore, 20 and 40 μM PCB could be used in subsequent in vitro experiments. Cells treated with IL-1β (10 ng/mL) showed lower viability compared to cells in the control group (0.2 % DMSO). After treatment with PCB2, the cells showed improved viability (P < 0.05) (Fig. 1D). EdU staining results showed that the proliferation of chondrocytes decreased after IL-1β treatment. However, PCB2 treatment significantly increased the ratio of proliferating cells in IL-1β-treated chondrocytes (Fig. 1E, F). These results suggest that PCB2 exerts a protective function on OA chondrocytes.

3.2. PCB2 attenuates the inflammatory reaction and ECM degradation of chondrocytes

Western blotting, RT-PCR, and immunofluorescence staining were performed to determine whether PCB2 might inhibit the IL-1β-triggered inflammatory response and ECM degradation in chondrocytes. PCB2 effectively inhibited IL-1β-triggered reduction in collagen II and aggregan expression. The results of immunofluorescence staining were consistent with those of western blotting (Fig. 2A–D). The effects of PCB2 were abolished by siRNA Nrf2 (Fig. 2E, F). These findings demonstrate that PCB2 markedly attenuated the inflammatory reaction and ECM degradation of chondrocytes while maintaining the homeostasis of cartilage.

3.3. PCB2 suppresses IL-1β-induced apoptosis in chondrocytes

The number of apoptotic chondrocytes increased significantly in the IL-1β-treated group. However, PCB2 treatment partially reversed this effect (Fig. 3A–C). The impact of PCB2 on markers of apoptosis was investigated using western blotting. Bax and caspase 3 were markedly upregulated in IL-1-stimulated chondrocytes, whereas Bcl-2 was notably downregulated (Fig. 3D, E). PCB2 pretreatment reversed these effects. Taken together, these results indicate that PCB2 significantly inhibited apoptosis (Fig. 3D, E). Targeted siRNA against Nrf2 reduced Nrf2 expression and inhibited the anti-apoptotic action of PCB2 (Fig. 3F–I).
These findings indicate that PCB2 has a strong anti-apoptotic role, which may be mediated through the Nrf2/BAX/Bcl-2 signaling pathway.

3.4. Effects of PCB2 on IL-1β-induced expression of SASP factors and senescence phenotype in rat chondrocytes

The effects of PCB2 on the mRNA, as well as protein levels of SASP factors were then assessed using western blotting, qPCR, and ELISA. As depicted in Fig. 4A, IL-1β-stimulated chondrocytes displayed considerably increased mRNA expression levels of MMP3, MMP10, IL-6, and IL-8 (p < 0.05), whereas the expression levels of MMP10, MMP3, IL-6, and IL-8 were markedly decreased in the PCB2-pretreated group in a dose-dependent manner (p < 0.05). Likewise, western blotting results revealed that PCB2 downregulated the p16, p21, MMP3, MMP10, IL-8, and IL-6 concentrations (p < 0.05; Fig. 4B–F). The ELISA results also verified these findings (Fig. 4G). The increased frequency of SA-β-gal-positive cells showed that IL-1β treatment caused senescence in chondrocytes, whereas PCB2 administration inhibited senescence induced by IL-1β treatment (Fig. 4H, I).

3.5. PCB2 inhibits activation of the NF-κB pathway

In order to explore the potential anti-inflammatory mechanism of PCB2, immunofluorescence and western blotting assays were used to identify biomarkers of the NF-κB pathway (p65 and IκBα). Immunofluorescence data revealed that p65 translocated from the cytoplasm to the nucleus, whereas PCB2 treatment inhibited p65 translocation (Fig. 5A, B). IL-1β treatment triggered a statistically significant increase in the ratio of p-p65/p65 and the amount of IκBα protein in chondrocytes (p < 0.05) (Fig. 5C–E). In contrast, pretreatment with PCB2 markedly lowered p-p65/p65 and IκBα protein levels. Meanwhile, we isolated the nucleus from chondrocytes and analyzed the expression of p65 in this organelle using western blotting. The data indicate that PCB2 suppresses the expression of p65 in the nucleus (Fig. 5C–E).

3.6. Molecular docking indicates the interaction between PCB2 and Nrf2

The three-dimensional (3D) model of PCB2 (Fig. S2A). In present research, PCB2 inhibited activation of the NF-κB pathway in IL-1β-
induced Chondrocytes. Many upstream molecules, including Nrf2, have been shown to influence the NF-κB pathway. The three-dimensional model of Nrf2 was shown in Fig. S2 B. In order to confirm the ability of PCB2 to activate Nrf2, a 3D binding model was used to exhibit the binding sites and affinity of PCB2 and Nrf2. The results showed that PCB2 might interact with the Nrf2 by hydrogen bonds, salt bridge, Alkyl and van der Waals, which contains three potential active sites (ARG-66, GLU-59, TYR-913) (Fig. S2 C). It also showed that PCB2 against the Nrf2 acquired binding affinity value (~8.7 kcal/mol), which shows that PCB2 has the high affinity with Nrf2 (Fig. S2C-D). These results imply that PCB2 might directly induct Nrf2 by binding to potential targets on Nrf2.

3.7. PCB2 regulates NF-kB signaling through Nrf2

Immunofluorescence staining data illustrate that PCB2 treatment enhanced Nrf2 nuclear translocation relative to IL-1β-treated and control groups (Fig. 6A). The expression levels of Nrf2 and HO-1 in the IL-1β treatment and control groups were not significantly different (Fig. 6B, C). However, PCB2 promoted the expression of Nrf2, which elevated the expression of Nrf2 in the nucleus and HO-1 in all cells (Fig. 6B, C),
consistent with the immunofluorescence data.

Chondrocytes were transfected with Nrf2-siRNA and then treated with IL-1β and PCB2. As illustrated in (Fig. 7A, B), Nrf2 siRNA suppressed the activation of the Nrf2/HO-1 signaling pathway. However, western blotting data following Nrf2 siRNA transfection revealed an increase in the p-p65/p65 ratio (Fig. 7C, D), demonstrating that PCB2 modulates NF-κB signaling through Nrf2. Furthermore, western blotting findings revealed that Nrf2 siRNA transfection increased p16, p21,
Fig. 4. Effects of PCB2 on the SASP factor expression and senescence phenotype in IL-1β-treated chondrocytes. (A) mRNA levels of SASP factors, including IL-6, IL-8, MMP3, and MMP10, were determined in chondrocytes treated with or without PCB2 and IL-1β for 24 h. (B and C) Expression levels of p21 and p16 proteins, assessed via western blotting, in chondrocytes. (D–F) Expression levels of SASP factors, assessed via western blotting, in chondrocytes. (G) MMP10 and IL-6 levels in the cell supernatants, assessed via ELISA. (H and I) SA-β-gal staining. (scale bar: 50 μm). Data are shown as mean ± SD. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.001.
MMP3, MMP10, IL-6, and IL-8 levels in transfected cells relative to their levels in PCB2-treated chondrocytes, (Fig. 7E–I), whereas the number of SA-β-gal-positive cells markedly increased (Fig. 7J, K). These findings indicate that PCB2 modulates senescence via Nrf2.

3.8. PCB2 has protective effects during the development of OA

A surgically rat DMM model was established to assess the therapeutic impact of PCB2 on the development of OA. Rats were divided into three different groups: sham, DMM, and DMM + PCB2. The number of chondrocytes decreased, chondrocytes were replaced by fibrochondrocytes, the ECM was lost, and the cartilaginous endplate collapsed in the DMM group; PCB2 treatment mitigated these degenerative effects. At 8 weeks, the OARSI scores of the sham, DMM, and DMM + PCB2 groups were 3.1 ± 0.6, 10.3 ± 1.5, and 5.2 ± 0.8, respectively. The OARSI scores of the DMM group was considerably higher than that of the sham group (P < 0.05). The OARSI score of the DMM + PCB2 group was lower than that of the DMM group (P < 0.05, Fig. 8A, B). Immunohistochemical staining indicates that PCB2 reduced the expression of p-p65 and increased the expression of Col-II and Nrf2 in the joints of the DMM group (Fig. 8C–H). Furthermore, immunohistochemical staining indicates that PCB2 reduced the expression of Bax and SAPA (p16, p21 and MMP13) and increased the expression of Bcl-2 (Fig. S1). Therefore, our results show that PCB2 attenuated the degeneration of the articular cartilage in the DMM rat model.

4. Discussion

OA is a common age-linked condition marked by cartilage degradation, synovial inflammation, as well as subchondral sclerosis [28,29]. Knee pain has also been linked to OA. Chondrocyte senescence and inflammation play a role in the pathogenesis of OA. Anti-inflammatory drugs, such as opiates and nonsteroidal anti-inflammatory drugs, which are often used in clinical practice to treat OA, are inadequate for reversing cartilage deterioration and may have harmful side effects if taken for long periods [30,31]. Therefore, novel therapeutic drugs with minimal adverse effects and excellent anti-inflammatory activity are required. Proanthocyanidins are the most commonly found flavonoid molecules in grape seeds and have anti-aging and anti-inflammatory properties [25]. We found that PCB2 reduced IL-1β-triggered SASP and apoptosis in chondrocytes and slowed the progression of OA by activating the Nrf2/HO-1 signaling pathway, indicating that PCB2 is a potential therapeutic drug for OA treatment. We demonstrated that PCB2 treatment decreased joint deterioration in a rat OA model. Inflammatory factors, particularly IL-1β, accelerate the course of OA by increasing chondrocyte death and senescence [32–35]. Furthermore,
IL-1β regulates SASP (MMP3, MMP10, IL-6, and IL-8) and leads to the production of various downstream factors [36]. Chondrocyte senescence is mediated by excessive IL-1β levels [17]. Therefore, IL-1β was used for in vitro stimulation in this study. Although the anti-aging and anti-inflammatory effects of PCB2 in OA are unknown, removal of senescent cells may slow the development of the disease. In present study, we established that PCB2 reduced IL-1β-triggered SASP in chondrocytes and slowed OA development by activating Nrf2, indicating that PCB2 is a potential therapeutic agent for the treatment of OA.

Chondrocytes rely on the ECM to respond to mechanical stresses. Collagen II and aggrecan are the most common ECM anabolic proteins. According to our findings, PCB2 enhanced the expression of collagen II and aggrecan in IL-1β-stimulated chondrocytes.

Importantly, p16 expression is strongly linked to age, and cellular p16 content has been suggested as a marker of cellular senescence [37]. The selective elimination of p16-high cells may enhance the longevity and health span of mice, suggesting that p16-expressing cells affect the development of age-linked pathologies [38]. In rat and human articular chondrocytes, elevated p16 expression is associated with age [39]. Our results show that PCB2 efficiently repressed P16 expression in chondrocytes.

The NF-κB signaling pathway is involved in the modulation of cell survival, proliferation, and differentiation, playing a pivotal role in inflammation, cell proliferation, oxidative stress, apoptosis, and senescence [40,41]. Under normal conditions, NF-κB usually binds to IκBα as a homologous or heterologous dimer to form a stable NF-κB/IκBα complex, which is inactive in the cytoplasm. IκBα masks the nuclear localization sequence of the NF-κB dimer and prevents it from entering the nucleus. IκBα may be phosphorylated and degraded in response to IL-1β stimulation, resulting in the translocation of the nuclear factor kappa-B p65 subunit (p65) from the cytoplasm to the nucleus and activation of particular genes [41]. Overexpression of SASP-linked genes in senescent cells is associated with NF-κB signalling [42]. In this study, PCB2 blocked the NF-κB signaling pathway by reducing the p-p65/p65 ratio and reversing p65 translocation to the nucleus. Many upstream molecules, including Nrf2, influence the NF-κB pathway [43]. PCB2 increases the viability of chondrocytes by Nrf2/NF-κB pathway.

Nrf2 is a critical component of the intracellular oxidative stress response and has anti-inflammatory, antitumor, and neuroprotective properties. Nrf2 is a crucial transcription factor found mostly in the cytoplasm that protects cells from oxidative stress [44]. Nrf2 forms hydrogen bonds with Keap-1 residues to maintain its stability. When Nrf2 is activated, it may separate from Keap-1, enter the nucleus, and bind to antioxidant-responsive sites, upregulating functional genes, such as HO-1. The Nrf2 pathway inhibits apoptosis, SASP-linked cellular senescence, and the NF-κB pathway during inflammation [43]. In this study, we found that IL-1β stimulation did not affect Nrf2 or HO-1 expression in chondrocytes. The expressions of Nrf2 and HO-1 was

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**Fig. 6.** Effects of PCB2 on Nrf2 signaling in IL-1β-stimulated chondrocytes. (A) Immunofluorescence images showing Nrf2 expression levels (scale bar: 20 μm). (B and C) Expression levels of nuclear Nrf2 and HO-1 proteins detected via western blotting. Data are shown as mean ± SD. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.001.
Fig. 7. Silencing of Nrf2 abolishes the anti-SASP effects of PCB2. (A and B) After Nrf2 siRNA transfection, the expression of Nrf2 and HO-1 proteins assessed via western blotting. (C and D) After Nrf2 siRNA transfection, expression of p-p65 and p65 proteins assessed via western blotting. (E-F) After Nrf2 siRNA transfection, expression of p21 and p16 proteins assessed via western blotting. (G-I) After Nrf2 siRNA transfection, expression of IL6, IL8, MMP3, and MMP10 proteins assessed via western blotting. (J-K) After Nrf2 siRNA transfection, SA-β-gal staining was detected. (scale bar: 50 μm). Data are shown as mean ± SD. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.
upregulated in a dose-dependent manner following pre-treatment with PCB2. Loss of Nrf2 increases NF-κB activity and boosts cytokine production [45]. We also introduced another Nrf2 siRNA to exclude the possibility of off-target effects. We used siRNA Nrf2 to transfet chondrocytes. The anti-senescence effects of PCB2 were abolished by siRNA Nrf2, which resulted in an elevated p-p65/p65 ratio and enhanced production of inflammatory mediators. These findings indicate that PCB2 controls senescence via Nrf2. PCB2 therapy also repressed IL-1β-induced apoptosis in chondrocytes. Furthermore, silencing Nrf2 partially reversed apoptosis-reducing effects of PCB2. These findings suggest that PCB2 rescues chondrocytes from IL to 1β-induced apoptosis via the Nrf2 pathway. In IL-1β-stimulated chondrocytes, these observations revealed that the anti-senescence action of PCB2 was mediated via Nrf2/HO-1 and NF-κB signaling pathways. Furthermore, PCB2 inhibited chondrocyte apoptosis via the Nrf2/BAX/Bcl-2 pathway. Although we established that regular use of PCB2 may slow the progression of OA, it is still unclear whether the effects of PCB2 on OA are direct or indirect. We believe that PCB2 has both direct and indirect effects on the chondrocytes. PCB2 may diffuse to the knee joint and reduce the senescence phenotype directly in chondrocytes, as described above. Simultaneously, tissues in the body communicate with one another via released cytokines, chemokines, and other proteins. Therefore, PCB2 may have an indirect impact on the secretory phenotype in tissues other than the knee joint. However, there are possible limitations of this study that should be addressed in future research. First, the impact of PCB2 on OA was only studied in rats, not humans. Second, a clinical study of the impact of PCB2 on OA is required.

5. Conclusion

The effects of PCB2 and its possible mechanisms on OA were explored in the present study. PCB2 enhanced ECM stability and inhibited the IL-1β-induced SASP phenotype and apoptosis in chondrocytes. The anti-senescence activity of PCB2 in OA was mediated via the Nrf2/NF-κB signaling pathway, as shown by a mechanistic assessment. We also established that PCB2 exerted anti-apoptotic effects via the Nrf2/BAX/Bcl-2 pathway (see Fig. 9). In addition, inoculation with PCB2 blocked OA progression in a rat OA model, suggesting that PCB2 is a prospective therapeutic agent for OA. Therefore, our findings suggest that PCB2 may be potentially used as a therapeutic agent for OA.

Author contributions.

HP, YZ, and WC worked on conception and design. In vivo experiments were conducted by WC, WJ, and YZ. WC performed the in vitro experiments and wrote the paper. CZ, SW, JW, and YZ contributed to the...
experimentation. HP, CT and WJ supervised the project and revised the manuscript. All authors read and approved the final manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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References


Fig. 9. Schematic illustration of the potential protective effects of PCB2 against the progression of OA. PCB2 ameliorates the SASP phenotype via the Nrf2/NF-κB axis and reduces IL-1β-induced apoptosis via the Nrf2/BAX/Bcl-2 axis in the progression of OA.


