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Chondroprotective effects of palmatine on osteoarthritis in vivo and in vitro: A possible mechanism of inhibiting the Wnt/β-catenin and Hedgehog signaling pathways

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ABSTRACT

The present study aimed to investigate the effect of palmatine (Pal) in a rabbit osteoarthritis (OA) model in vivo and rabbit interleukin-1β (IL-1β)-stimulated chondrocytes in vitro. Appropriate concentrations of Pal were identified by the MTT assay and used to preincubate IL-1β-induced chondrocytes, as well as an activator or inhibitor of Wnt and Hedgehog signaling pathways. Matrix metalloproteinase (MMP)-1, 3, and 13; tissue inhibitor of metalloproteinase (TIMP)-1; collagenase II; aggrecan; and the related molecules of the Wnt/β-catenin and Hedgehog signaling pathways were investigated. Protein expression was detected by Western blot analysis and messenger RNA (mRNA) expression was examined by PCR analysis. Pal (0.3 mL, 100 mg/L) was injected into rabbit knee joints and histological examination, immunohistochemistry, and Mankin scoring of the articular cartilage were performed. Pal (10–100 mg/L) had no effect on chondrocyte viability, decreased the expression of the MMPs, and increased the synthesis of TIMP-1whereas collagenase II and aggrecan were inhibited by IL-1β. When the activator (Licl) and inhibitor (DKK-1) of the Wnt/β-catenin signaling pathway as well as the inhibitor (cyclopamine) of the Hedgehog signaling pathway were added, the Wnt/β-catenin signaling pathway was less inhibited by Pal, and a similar inhibitory effect of cyclopamine on the Hedgehog signaling pathway was evident. Additionally, Pal enhanced the effect of cyclopamine. The histological examination, immunohistochemistry and Mankin scoring also demonstrated the protective effect of Pal, and the inhibition of the Wnt and Hedgehog signaling pathways by Pal. Pal may be useful in the treatment of OA, in which its effect is likely mediated via the Wnt/β-catenin and Hedgehog signaling pathways.

1. Introduction

Osteoarthritis (OA) is the most prevalent form of joint disease and evolves from a local inflammatory response caused by joint instability due to a chronic process accompanied by progressive degeneration of articular cartilage [1]. Reportedly, more than 12% of the aging Western population suffers from this disease, especially those 45 years of age and older [2,3]. Obesity, trauma, genetic factors, bone mass and instability, developmental diseases, joint injury, joint deformity, and age are considered common causes of OA, especially in the knee joints [4,5].

Although OA is regarded as primarily a non-inflammatory arthropathy, symptoms of local inflammation as well as synovitis are present during the course of cartilage destruction. When OA occurs, chondrocytes produce excessive amounts of proteolytic enzymes, mainly matrix metalloproteinases (MMPs) and collagenases, aggrecanases and nitric oxide (NO) in response to proinflammatory stimuli such as interleukin-1β(IL-1β) and tumor necrosis factor alpha (TNF-α) [6]. The MMP family is considered a major factor in the pathophysiology of OA. Among the MMPs, MMP-1, MMP-3, and MMP-13 play a central role in cartilage degradation in OA. Tissue inhibitors of metalloproteinases (TIMPs) act as inhibitors of MMPs. TIMP levels are elevated in OA cartilage, possibly reflecting an endogenous adaptive response to the increased levels of proteinase activity [7]. In addition, several novel signaling pathways have been recognized in OA, such as Wnt and hedgehog, which play key roles in skeletal development, including chondrogenesis via the regulation of cell proliferation, differentiation, survival and migration [8–12].

Although many drugs are currently approved for treating this illness, none has been definitely showed to slow the structural progression of OA. Palmatine (Pal), a member of the protoberberine class of isoquinoline alkaloids, is a structural analog of berberine [13]. Pal is an important traditional medicinal compound and a bioactive herbal

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ingredient isolated from *Rhizoma coptidis*, *Cortex phellodendri*, *Radix tinosporae* and *Enantia chlorantha* [14]. This alkaloid has been used in the treatment of jaundice, hypertension, dysentery, inflammation, and liver-related diseases. Pal was shown to possess antipyretic, antibacterial, antifungal, antiviral, antiphotooxidative, dampness-dispelling, antidote, antinociceptive, and anti-inflammatory properties in vitro and in vivo [15–20]. Other studies focused on the molecular mechanisms related to signaling pathways, such as the Raf/MEK1/ERK1/2 [21] and growth factors-MAP-kinase pathways [22]. The anti-inflammatory activity of Pal implies a possible therapeutic effect on inflammatory-mediator-related diseases [23]. However, the effects of Pal on chondrocytes and OA remain unclear. Therefore, the present study investigated whether Pal could slow down the progression of OA via the Wnt/β-catenin and Hedgehog signaling pathways by assessing the effects of Pal in an in vivo OA rabbit model and rabbit chondrocytes in vitro.

2. Materials and methods

2.1. Reagents

For the present study, Pal, recombinant human IL-1β, Dickkopf-1 (DKK-1), lithium chloride (Licl) and cyclopamine were obtained from Sigma-Aldrich (St. Louis, Mo, USA), and Dulbecco's modified Eagle's medium (DMEM), D-Hanks Balanced Salt Solutions, penicillin, streptomycin, fetal bovine serum (FBS), 0.25% trypsin, and collagenase II were purchased from Gibco BRL (Grand Island, NY, USA). Pal was dissolved in D-Hanks Balanced Salt Solutions and diluted with DMEM for both the in vivo and in vitro experiments.

2.2. In vitro experiments

Animal care and treatment were in accordance with the Guidelines of the Laboratory Animal Management and the Institutional Animal Care and Use Committee of Zhejiang University (Hangzhou, China).

2.2.1. Isolation and culture of chondrocytes

In this study, 4-week-old New Zealand white rabbits were sacrificed. Under sterile conditions, articular cartilage isolated from the knee joints of rabbits was digested with 0.25% trypsin for 30 min to remove other tissues, and then digested again with 0.1% collagenase II in DMEM with 10% FBS at 37 °C for 4 h. Cells were grown to confluence in DMEM supplemented with 10% FBS and antibiotics (100 U/mL penicillin and 100 mg/mL streptomycin) at 37 °C with 5% CO_2. After the cells were cultured up to approximately 80–90% confluency, confluent primary chondrocytes were passaged at a ratio of 1:3. Chondrocytes passages twice were used [10].

2.2.2. Chondrocyte viability assay and treatment

Chondrocytes were cultured in a 96-well plate (5000/well). After separately incubating the cells with various concentrations of Pal (10–200 mg/L) for 24, 48, or 72 h in a serum-free medium, MTT (5 mg/mL) was added to each well (20 μL/well). The cells were incubated with MTT for 4 h, the culture medium was removed and dimethyl sulfoxide (DMSO) was added to each well (150 μL/well). Absorbance was measured at 570 nm, and six duplications were used for each concentration. The chondrocytes were seeded in six-well plates (2×10^5/well), and the subconfluent cells were preincubated with four concentrations of Pal (10, 25, 50, and 100 mg/L as determined in preliminary tests) for 1 h followed by stimulation with IL-1β (10 ng/mL) for 24 h. The cells were then harvested, and the related gene mRNA expressions and protein levels were determined to assess the Pal concentrations, which were then used in subsequent experiments. Additionally, an activator (Licl) and an inhibitor (DKK-1) of the Wnt signal pathway and an inhibitor (cyclopamine) of the Hedgehog signal pathway, were applied for 1 h after stimulation with IL-1β, to explore the association between Pal and these two signal pathways.

2.3. In vivo tests

The present study included 21 4-week-old New Zealand rabbits weighing 2.0–2.5 kg (Animal Centre of Zhejiang University). Of the 21 rabbits, 14 underwent bilateral anterior cruciate ligament transections (ACLTs) on the knee joints to induce OA and were randomly divided into two groups (Pal group and OA group). The remaining seven rabbits (sham group) received sham operations, that involved opening the articular cavity and re-suturing it without cutting the short ACL. Following the surgery, all animals were returned to their cages without immobilization of the limbs, and 400,000 units of penicillin were injected intramuscularly for 3 days. One month after surgery, the Pal group was given intra-articular injections of Pal (0.3 mL, 100 mg/L) in both knees once per week for 5 weeks, the OA group was injected with 0.3 mL of D-Hanks Balanced Salt Solutions alone in both knees under the same conditions, and no other procedures were conducted in the sham group. All rabbits were sacrificed 7 days after the final injection.

2.4. Real-time polymerase chain reaction (PCR) analysis

Total RNA was isolated from the chondrocytes and cartilage treated with Pal using the Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. First-strand cDNA synthesis was achieved using a primescript-RT reagent kit (TaKaRa Biotechnology Co., Ltd., Japan). Target gene mRNA levels were determined by a real-time PCR procedure using SYBR Premix Ex Taq (TaKaRa) as follows: 30 s at 95 °C for the initial denaturation, followed by 40 cycles at 95 °C

Table 1
Primers of targeted genes.

	Forward	Reverse
MMP-1	5′-ATGCTGAAACCCTGAAGATGAT-3′	5′-CCTTGGAGACTTTGGTGAATGT-3′
MMP-3	5′-CGTTCCTGATGTTGGTCACTT-3′	5′-TCAGCCTCTCCTTCATACTTCC-3′
MMP-13	5′-CCCCTCCTCAACAGTAACGAG-3′	5′-AGTTTGCCTGTCACCTCTAAGC-3′
TIMP-1	5′-CAACTGCGGAACGGGCTCTTG-3′	5′-CGGCAGCGTAGGTCTTGGTGAA-3′
β-catenin	5′-CGTACGCACCATGCAGAACACAAA-3′	5′-ATCCACTGGTGAACCGAGCATCTT-3′
DKK-1	5′-TCAAGTGTGCACCAAGCACAGGA-3′	5′-ATCGTTCTGCAGCCGACAAGACA-3′
GSK-3β	5′-GGACTAAGGATTCGTCAGGAACAG-3′	5′-GTGGTGTCAGTCGGGCAGTT-3′
Ihh	5′-GGCCATCTCCGTGATGAACCAGT-3′	5′-CGCGCCAGCAGTCCGTACTTATT-3′
Shh	5′-CGTCTGACCGTGACCGTAGCAA-3′	5′-GGACTCATAGTAGACCCAGTCGAA-3′
Gli-2	5′-GCCCACGAGCACCTGAAAGAGA-3′	5′-CAGCCATGAGGGTCATCTGATGGTA-3′
Collagen II	5′-GACTGCCTGAGCCCCGAGAT-3′	5′-CTGCCCCTTTGGTCCTGGTTTC-3′
Aaggrecan	5′-ATGGCTTCCACCAGTGCG-3′	5′-CGGATGCCGTAGGTTCTCA-3′
TNF-α	5′- GTGACGAGCCTCTAGCCCACGTAGT-3′	5′- GACCGCTGAAGAGAACCTGGGAGTA-3′
18S rRNA	5′-CGTAGTTCCGACCATAAACGAT-3′	5′-AATCTGTCAATCCTGTCCGTGT-3′