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원 저

The Effects of *Betula Platyphylla* on Cartilage Protection, Anti-inflammatory and Analgesic Activity in Arthritis

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국문초록

관절염 유발 모델에서 화피가 연골 보호 및 소염 진통에 미치는 영향

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목적 : 본 연구는 화피의 연골 보호 및 소염 진통 작용을 알아보고, 화피를 이용한 관절염 치료 약침액 개발의 기초자료를 얻기 위해 고안되었다.

방법 : *In vitro*에서는 토끼 무릎관절에서 배양된 연골조직에 5ng/ml IL-1α 처리 후, 화피의 연골보호 효과, 연골세포에 대한 독성을 조사하였다.

*In vivo*에서는 토끼 무릎관절내 collagenase를 주입, CIA 유발 후, 28일간 매일 토끼의 구강으로 화피, 증류수, CEX를 투여하였으며, 연골보호, 소염, 진통에 대한 측정을 하였다.

결과 : 화피는 proteoglican 및 collagen분해 억제, MMPs 활성 억제로 연골 보호 효과가 있었으며, 연골세포에 대한 독성이 없었다. 소염작용은PGE2생산 억제 및COX-2발현 억제, carrageenan 유발 쥐 모델에서의 부종 억제로 확인되었다. 진통작용은 tail flick test에서의 latency time증가, formalin test에서의 염증성 통증 억제로 나타났다.

결론 : 화피가 퇴행성관절염에 대한 연골 보호 효과 및 소염 진통 작용이 있으므로, 이를 근거로 약침액을 개발 응용하면 퇴행성관절염 치료에 활용될 수 있다고 사료된다.

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핵심단어 : *Betula Platyphylla*, osteoarthritis, cartilage, anti-inflammation, analgesia, collagenase-induced arthritis; CIA

Ⅰ. Introduction

Osteoarthritis(OA) is a multifactorial degenerative joint disease in which the cartilaginous matrix of articular joint is destroyed. In the normal articular joint, cartilage homeostasis is maintained by a balance between cytokine - mediated anabolic and catabolic processes[1-3]. However, in OA, the balance shifts toward catabolism, leading to cartilage destruction. In general, the destruction of cartilage in OA is initially caused by a decrease in its proteoglycan content, followed by the degradation of collagen fibers. Some studies have suggested that investigation into cartilage degradation should include an examination of both proteoglycan and the collagen matrix.[4,5]

An ideal therapeutic agent for OA, in addition to decreasing pain and inflammation, protection against cartilage destruction is the ultimate goal for OA treatment; however, current pharmacological therapies remain unsatisfactory[6,7]. The non-steroidal anti-inflammatory drugs(NSAIDs) inhibit early steps in the biosynthesis pathway of prostaglandins by inhibition of cyclooxygenase(COX) enzymes and are the main drugs used to reduce the further consequences of inflammation[8]. However, NSAIDs cause several serious adverse effects due to their non-selective inhibition of both isoforms of the COX enzyme[9-12].

The bark of *Betula platyphylla*(BP) has been used in traditional medicine for the treatment of arthritis, cancer, nephritis, dermatitis, poisoning, and chronic bronchitis. Ju et al.[13] reported that an extract of BP has antioxidant and anticancer activity. Recently, some of the herbal acupuncture solutions are being applied to osteoarthritis clinically.

In this study, we attempted to get a source material for the development of new herbal acupuncture solution of BP for OA. The effect of BP on cartilage protection, anti-inflammatory and analgesic activity was observed in vitro and in vivo.

Ⅱ. Materials and Methods

1. *In Vitro*

1) Preparation of BP extract solution

Bark of *BP* (500g) was obtained from Kyunghee University Hospital of Oriental Medicine. The BP was incubated in 50% (v/v) ethanol - water at room temperature for 24 h. The concentrate was freeze-dried and its yield was 12.1% (60.5 g).

2) Cartilage explants culture

Articular cartilages were obtained from the joints of five-week-old male rabbits (Samtako Biokorea Co., Korea). The articular surfaces were surgically exposed under sterile conditions; approximately 200 - 220mg of articular surface per joint was removed and steeped in complete medium.

3) Glycosaminoglycan(GAG) degradation assay

GAG levels in the culture medium were determined by the amount of polyanionic material reacting with 1,9-dimethylmethylene blue, using shark chondroitin sulfate as the standard.

4) Collagen degradation assay

Type II collagen levels in the culture medium were determined using the Sircol Collagen Assay.

5) Colorimetric analysis of matrix metalloproteinases (MMP) activity

The levels of MMP activity in the conditioned media were evaluated using an enzyme-linked immunosorbent assay(ELISA) kit.

6) Measurement of lactate dehydrogenase (LDH) activity

As an indicator of cell viability, the cytoplasmic enzyme LDH was measured in the culture medium. An optimized LDH test was used to quantify LDH activity in the medium of the cartilage explants cultures.

7) Statistical analysis

The results were expressed as means ± S.D. calculated from the specified numbers of determinations. Statistically significant differences relative to the untreated control group were calculated by Student's one-tailed paired t test. Differences with p values < 0.05 were deemed statistically significant.

2. *In Vivo*

1) Preparation of BP extract solution

Bark of *BP* (2000g) was purchased from Kyunghee University Hospital of Oriental Medicine. *BP* was extracted with 50% (v/v) ethanol - water at 60°C for 8 h. The concentrate was freeze-dried and its yield was 12.1% (242g).

2) Induction of collagenase - induced arthritis (CIA)

New Zealand white male rabbits were obtained from the animal experimental center (Samtako Biokorea Co., Korea) and individually housed. Rabbits weighing 2.8-3.0kg(aged 9-10 weeks) at the start of experiments (1st day) were anesthetized with an intra-muscular injection of tiletamine-zolazepam (Zoletil50, Virbac, France). The shaved right knee joints of all rabbits were injected intraarticularly with 250㎕ of 4 mg/ml collagenase solution (Clostridium histolyticum, type II; enzyme activity 425U/mg) or saline as a negative control. The same collagenase injection procedure was applied once more on 4th day according to the method of Mankin. Following the initial injection of collagenase (1st day), the rabbits were divided into control and experimental group (n = 10 per group). For 4weeks, groups were orally treated with 20㎖ distilled water (DW), celecoxib (CEX 100mg/kg) and *BP* (200mg/kg) once a daily base using feeding catheter (DJ2-284, Dae jong Ins. Korea).

3) Carrageenan-induced paw edema

The edema inducing agent, i.e. 0.1 ml of 1% carrageenan in normal saline was injected into the plantar surface of left hind paw after 30min of oral administration. The volumes of injected paws were measured before, 60, 120 and 180min after injection of carrageenan using Ugo Basile plethysmometer.

4) Tail flick test

The tail flick test was performed with the Tail-Flick Analgesia Meter. The tests were performed at 60min after oral administration.

5) Formalin test

After 30min of oral administration, 50㎕ of 5% formalin was injected subcutaneously into the dorsal surface of the right hind paw with a 30-G needle.

6) Statistical analysis

Data were expressed as means ± SEM and differences between mean values were analyzed by unpaired Student's t test. p values less than 0.05 or 0.01, which were calculated as one tailed p values, were considered statistically significant.

Ⅲ. Results

1. Effect on Cartilage Protection of Articular Cartilage Explants in Rabbit

1) Effect on proteoglycan and collagen degradation

BP dose-dependently reduced IL-1α-mediated GAG and collagen release into the culture medium (Fig. 1).

2) Effect on MMPs expression and activities

BP inhibited MMP-3 and MMP-13 mRNA synthesis at 14days of culture in a dose-dependent manner. MMP-3 and MMP-13 levels decreased dose-dependently in the culture media with BP at 14th day(Fig. 2).

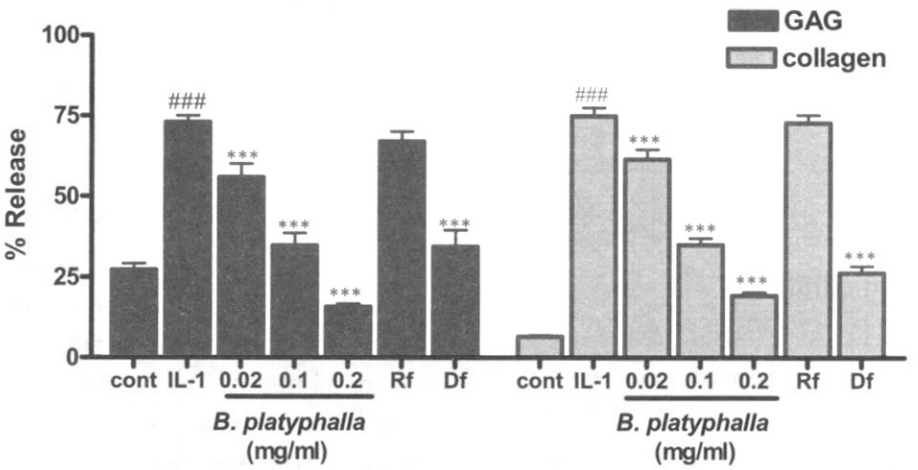

Fig. 1. Effect of BP on the dose response of proteoglycan and collagen degradation in rabbit cartilage explants cultures

Cartilage was cultured in quadruplicate in 400 $\mu\ell$ of medium only, with 5 ng/ml IL-1α, or with 5 ng/ml IL-1α plus different concentrations of BP or rofecoxib (Rf), or diclofenac (Df) for 14 days. $^{###}p < 0.001$ compared with non-treated group, and $^{***}p < 0.001$ compared with respective control (IL-1α).

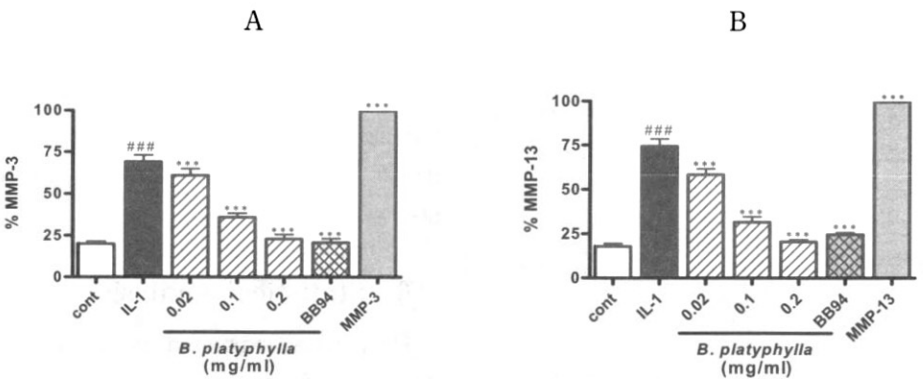

Fig. 2. Effect on MMP activity in rabbit cartilage explants cultures

A: MMP-3 activity B: MMP-13 activity.

$^{###}p< 0.001$ compared with non-treated group, and $^{***}p< 0.001$ compared with respective control (IL-1α).

3) Effect on the viability of cartilage explants

It was not detected that any LDH activity in the incubation medium of cultures, indicating that neither IL-1α nor *BP* has cytotoxic effects on chondrocytes cartilage explants during 3, 7, or 14 days of culture.

2. Effect on Cartilage Protection, Anti-inflammatory and Analgesic Activity *In Vivo*

1) Effect on cartilage protection in CIA

The proteoglycan was well preserved in *BP* group, but DW group resulted in a marked loss of proteoglycan. BP significantly reduced the release of GAG and collagen(Fig. 3).

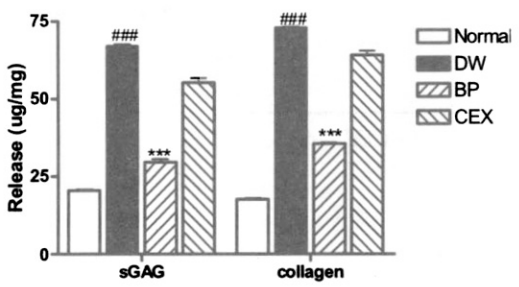

Fig. 3. Effect on cartilage protection against cartilage degradation in CIA
###p<0.001 compared with normal, ***p<0.001 compared with DW.

2) Effect on MMPs in CIA

The activity of MMP-1 and MMP-3 was rarely detected in the synovial fluid of normal rabbits, while there was a significant increase in DW group by more than 200-fold and 100-fold, respectively. *BP* significantly reduced the level of MMPs(Fig. 4).

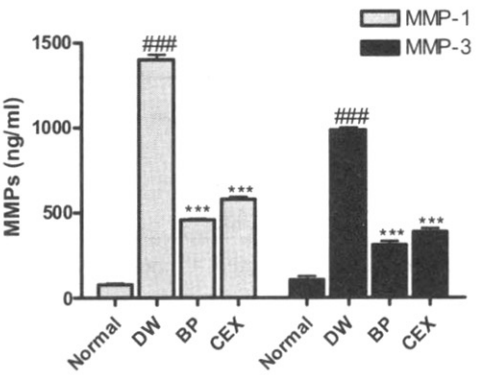

Fig. 4. Effect on the level of MMPs
###p<0.001 compared with normal, ***p<0.001 compared with DW.

3) Effect on PGE2, COX-1 and COX-2 in CIA

In *BP* group, PGE2 release was significantly reduced compared to DW group (Fig. 5A). COX-2 mRNA expression was significantly reduced compared to DW group(Fig. 5B).

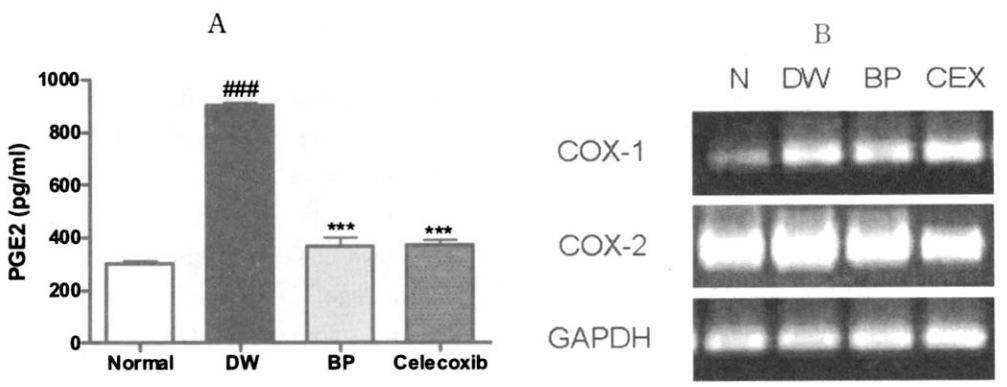

Fig. 5. Effect on the production of inflammation-related molecules
A: The inhibition of PGE2 production in joint extract from knee joints.
###p<0.001 compared with normal, ***p<0.001 compared with DW.
B: The levels of COX-1 and COX-2 mRNA expression.

Table 1. Inhibition of Carrageenan-Induced Rat Paw Edema

Group	Dosage	Inhibition (%) 1h	2h	3h
DW		80.4 ± 0.5*	89.6 ± 0.8*	80.4 ± 0.5*
BP	200mg/kg	134.3 ± 0.6**	127.8 ± 2.9*	121.1 ± 4.6*
CEX	100mg/kg	100.0 ± 2.9	100.0 ± 7.7	100.0 ± 9.9

Each datum was represented as mean ± S.D. (n=12) *p<0.05, and **p<0.01 compared to CEX group.

Table 2. Analgesic Effect in Tail Flick Test in Rats

Group	Dosage	Time of response (s)	Inhibition (%)
DW		5.7 ± 0.8*	81.4 ± 3.5*
BP	200mg/kg	8.9 ± 1.0*	121.1 ± 3.6*
CEX	100mg/kg	7.0 ± 0.7	100.0 ± 5.1

Each datum was represented as mean ± S.D. (n=12) *p<0.05 compared to CEX group.

Table 3. Analgesic Effect in Formalin Test in Rats

Group	Dosage	Inhibition (%) 1st phase	2nd phase
DW		87.7 ± 6.5*	90.0 ± 6.1
BP	200mg/kg	123.1 ± 6.3*	133.1 ± 4.6*
CEX	100mg/kg	100.0 ± 4.1	100.0 ± 5.2

Each datum was represented as mean ± S.D. (n=12) *p<0.05 compared to CEX group.

4) Effect on anti-inflammatory activity

BP effectively inhibited the increase of paw volume during the early phase and also exhibited a weak inhibition in the late phase(Table 1).

5) Effect on analgesic activity

The analgesic effect reflected in tail flick test is dependent on the centrally acting opioid - like analgesics. *BP* significantly increased the latency time(Table 2).

The analgesic activity of *BP* was also assessed by formalin test in rats. *BP* had a better analgesic activity than that of CEX in both early (1st phase) and late phase (2nd phase) of formalin induced nociception(Table 3).

Ⅳ. Discussion

OA is a degenerative joint disease characterized by progressive loss of articular cartilage, subchondral bond remodeling, spur formation, synovial inflammation, and in particular, the degradation of proteoglycan and collagen. The integrity of these macromolecules is vital to cartilage and joint function[5].

MMPs are synthesized in response to cytokines such as interleukin-1 (IL-1) and tumor necrosis factor-α, which is thought to be involved in the pathogenesis of arthritis[14-17]. MMPs were found likely to be the primary enzymes involved in the breakdown of GAG release and type II collagen in OA cartilage[18,19].

Two isoforms of COX, COX-1 and COX-2, catalyze the rate-limiting step in the conversion of arachidonic acid to prostaglandins and thromboxane A2 (TXA_2)[20]. COX-1 is a constitutively expressed protein that is thought to produce basal

concentrations of prostaglandins and TXA2, both of which are considered necessary for normal physiologic functions in many tissues. COX-2 is induced by cytokines in synovial tissue fibroblasts from arthritis patients and is thought to play a primary role in the pain and inflammation associated with that disease[21,22].

BP dose-dependently reduced IL-1α mediated proteoglycan release into the culture medium. *BP* markedly reduced collagen degradation after 14 days compared with that in IL-1α-treated cultures (Fig. 1). These results suggest that *BP* is effective for the reduction of proteoglycan and collagen degradation in rabbit cartilage. The control, diclofenac, a non-selective COX-2 inhibitor, showed inhibitory effect, whereas rofecoxib, a selective COX-2 inhibitor, didn't show any inhibitory effect(Fig. 1). *BP* dose-dependently inhibited MMP-3 and MMP-13 mRNA expression, and their activity in articular cartilage explants (Fig 2). *BP* had no impact on the viability of cartilage explants, when determined on the 3rd, 7th and 14th day of the culture period.

In vivo experiments indicated that the oral administration of *BP* can improve the degeneration of articular cartilage in OA. *BP* prevented the loss of proteoglycan and type II collagen and inhibited the expression of MMPs mRNA and activities of MMPs in CIA model(Fig. 3, 4).

BP also significantly inhibited COX-2 expression and PGE2 production (Fig. 5). It is suggested that *BP* might reduce enzyme activities for cartilage degradation, perhaps by suppressing the expression of inflammatory mediator and/or MMPs itself.

The presence of edema is one of the prime signs of inflammation. It has been documented that carrageenan-induced rat paw edema is a suitable *in vivo* model to predict the value of anti-inflammatory agents, which act by inhibiting the mediators of acute inflammation[23]. In the anti-inflammatory test, BP lowered the increase of paw volume (edema) than CEX(Table 1).

The tail flick test was applied as a model that has been used widely to study central algesic/analgesic action[24]. The formalin test was chosen as a simple two-phase model of tonic pain and localized inflammation. The early phase is considered to be a direct result of stimulation of nociceptors in the paw and reflects centrally mediated pain while the late phase, which is also termed as inflammatory pain, is a pain due to inflammation[25].

BP significantly increased the latency time in tail flick test(Table 2). *BP* had a better analgesic activity than that of CEX in both early and late phase of formalin induced nociception in rat (Table 3). However, the activity of *BP* extract was more pronounced in the late phase, which is commonly associated with inflammatory pain.

In summary, *BP* inhibited the cartilage destruction by inhibiting proteoglycan degradation, collagen degradation and MMPs activities both in vitro and *in vivo*. *BP* also had a significant correlation with anti-inflammatory and analgesic activities in experimental model. Therefore, these data suggest that *BP* has great potential in clinical application for cartilage protection and anti-inflammatory analgesic effect on osteoarthritis.

V. Conclusions

The effects of *BP* on cartilage protection, anti-inflammatory and analgesic activity were observed. IL-1α-induced rabbit cartilage explants and CIA model were used *in vitro* and *in vivo*, respectively. The results obtained are as follows.

1. *BP* showed the cartilage protection in vitro and *in vivo*, by inhibiting GAG release, collagen release and MMPs activities.
2. *BP* showed the anti-inflammatory activity *in vivo*, by inhibiting PGE2 production, COX-2 expression and reducing the paw edema of carrageenan-induced rat.
3. *BP* showed the analgesic activity *in vivo*, by

increasing the latency time in tail flick test and decreasing the number of paw flinches in formalin test.

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