Protective Effects of Human Placenta Extract on Cartilage Degradation in Experimental Osteoarthritis

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This study investigated the effect of human placenta extract (HPE) on cartilage degradation *in vitro* MG-63 cells, articular cartilage explants, and *in vivo* monoiodoacetate (MIA)-induced osteoarthritis (OA). Matrix metalloproteinase (MMP)-2 activity was measured in HPE-treated osteoblastic MG-63 cells. Articular cartilage explants in rabbit were cultured, and the degree of proteoglycan (PG) degradation was assessed by measuring the amount of glycosaminoglycan (GAG) released into the culture medium. Experimental osteoarthritis was induced by intra-articular injection of 3 mg MIA in rats. Beginning 14 d post-MIA injection, HPE was administered intra-articularly once a day for 14 d. The knee joints were assessed by roentgenography, histology, and gelatinase activity. HPE inhibited PG degradation in articular cartilage explants. HPE significantly reduced deformity of knee joints and suppressed the histological change in MIA-induced OA. HPE inhibited MMP-2 activity in MG-63 cells. MMP-2 and -9 activities were also reduced in the cartilages of HPE-treated knee joints. Our results indicate that HPE has therapeutic effects on OA by protecting cartilage.

Key words Laennec; human placenta extract; osteoarthritis; monoiodoacetate; glycosaminoglycan; matrix metalloproteinase

Osteoarthritis (OA) is a degenerative joint disease characterized by articular cartilage loss, subchondral bone remodeling, joint space narrowing and bone spur formation. The initiation of cartilage breakdown is stimulated with mechanical stress or injury, and the degenerative processes slowly progress over many years. In the advanced stage of OA, the abnormal remodeling of cartilage and subchondral bone results in the formation of osteophytes at the joint surface and margins, which irreversibly destroys the affected joint.[1)]

In the traditional medical view, OA has been seen as an inevitably progressive mechanical disorder; reparative approaches to therapy were therefore largely discounted. However, increasing knowledge of chondrocyte function and behavior suggests that drug therapy is very important and has various effects at the articular cartilage level. Over the last two decades, studies on OA have mainly focused on the effect of exogenous biochemical mediators on metabolism of articular cartilage. Intra-articular injection of hyaluronan, which is one of the principal components of cartilage matrix, is now frequently performed for the palliation of joint pain and has been reported to have some positive effects on OA maintenance.[2)] However, the mechanism of action of hyaluronan remains unclear.

Human placenta, a passage for nutrients, has been used in traditional oriental medicine for wound healing.[3)] Scientists studying placenta have found that it contains several enzyme inhibitors, anticoagulant proteins and antioxidants.[4—6)] Placental extract induced significant nitric oxide production in both rodent and human cells *in vitro*, which is an important cellular mediator of tissue repair.[7)] Recently, Honghwain-Jahage, the water extracts of *Carthamus tinctorius* L. seed and Hominis placenta, inhibited interleukin (IL)-1β-stimulated bone resorption in mouse osteoblast and osteoclast cells.[8)] Also, the immunoregulatory function of placenta, illustrated by suppression of cellular immunity and by attenuation of rheumatoid arthritis after injection into an acupuncture point,[9)] is another basis for its use in OA.

In this study, we used osteoblastic MG-63 cell line, rabbit *in vitro* cartilage explants and a rat *in vivo* monoiodoacetate (MIA)-induced OA model to investigate whether human placenta extract (HPE) has protective effects against cartilage degradation.

MATERIALS AND METHODS

Materials MIA and diclofenac were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). Dulbecco's modified Eagle's medium (DMEM), heat-inactivated fetal bovine serum (FBS), penicillin and streptomycin were purchased from PAA Laboratories GmbH (Pasching, Austria). MG-63 cells were obtained from American Type of Culture Collection (Manassas, VA, U.S.A.). Recombinant human interleukin-1α (rhIL-1α) was purchased from R&D Systems (Minneapolis, MN, U.S.A.). All other chemicals used in this study were reagent grade and were locally and commercially available.

Animals Male Sprague-Dawley rats (200—220 g) and male New Zealand white rabbits (2.0—2.2 kg) were obtained from Dae Han Biolink Ltd. (Korea) and housed in solid bottom cages with pellet food and water available *ad libitum*. The animal protocols used in this study were in accordance with the Sungkyunkwan University Animal Care Committee guidelines.

Preparation and Composition of Human Placenta Extract We used an HPE, Laennec, supplied by Green Cross Japan Bio Products Ltd., Korea. HPE is an aqueous extract of human placenta. Human placentas are collected upon full-term delivery and immediately placed in ice. They are tested for human immunodeficiency virus and hepatitis B and C viruses, then cut into pieces, and extracted with water through enzymatic molecular separation and chemical hydrolysis. Extraction and sterilization are performed subject to strict control. The final HPE products are stored at 2 ml/ampule. Each sample consists of various amino acids including arginine (0.08%), lysine (0.1%), phenylalanine (0.08%),

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tyrosine (0.03%), leucine (0.12%), methionine (0.03%), valine (0.04%), alanine (0.08%), serine (0.07%) and threonine (0.06%). Insoluble macromolecules, such as polysaccharides, polynucleotides, *etc.* are excluded during the manufacturing process.

***In Vitro* Study. Cell Culture** Cell lines were separately grown as monolayers in T-75 tissue culture flasks (Nunc, Roskilde, Denmark) at 5% CO_2 and 37 °C humidified atmosphere using appropriate media supplemented with 10% FBS, 2 mM glutamine and 100 μg/ml penicillin/streptomycin. DMEM was used as the culture medium for MG-63 (human osteosarcoma) cells.

Cartilage Glycosaminoglycan Assay Rabbit knee articular cartilage explants were obtained according to the method described by Sandy *et al.*[10] Briefly, 200—220 mg articular surfaces per joint were submerged into complete medium of DMEM supplemented with heat-inactivated 5% FBS, penicillin (100 U/ml) and streptomycin (100 μg/ml). After stabilization in incubator, the complete medium was replaced with basal medium made of DMEM supplemented with heat-inactivated 1% FBS, 10 mM *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid (HEPES), penicillin (100 U/ml) and streptomycin (100 μg/ml). Cartilage pieces (50—60 mg; 2×3×0.35 mm/piece) were placed in 24-well cell culture plates and treated with 1.8×10^{-2}, 1.8×10^{-1} and 3.6×10^{-1} ml HPE/ml media or 30 μM diclofenac. After 1 h pretreatment of HPE or diclofenac, 5 ng/ml of rhIL-1α was added to the culture medium and further incubated at 37 °C in a humidified 5% CO_2/95% air incubator. The control group was treated with PBS instead of rhIL-1α. The culture medium was collected 24, 48, 72, 96 and 120 h later and stored at −20 °C until use. The amount of glycosaminoglycan (GAG) in the medium was determined by 1,9-dimethylmethylene blue method using the Blyscan Sulfated GAG Assay kit (Biocolor Ltd., County Antrim, U.K.) according to the manufacturer's instructions.

***In Vivo* Study. MIA-Induced Osteoarthritis** MIA was dissolved in physiologic saline and administered in a 50 μl volume. Rats were anesthetized with diethyl ether and given a single intra-articular injection of 3 mg MIA through the infrapatellar ligament of the left knee.[11] Rats were then left in their cages for 14 d to induce appropriate OA. After 14 d, MIA-injected rats were divided into six groups. Each group was treated with saline, with 0.022, 0.066, 0.2 or 0.4 ml/kg of HPE or with 3 mg/kg of diclofenac by intra-articular injection once daily for 14 d. These HPE doses and injection volume were selected based on previous evaluation by Yeom *et al.*[9] To investigate the early biomolecular event, HPE or diclofenac was administered intra-articularly 6 and 30 h after MIA injection. Forty eight hours after MIA injection, the animals were sacrificed and their articular cartilages were stored for further assays.

Gross Observation After MIA injection, all experimental rats were weighed and carefully inspected every 2 d to assess swelling of their knee joints and gait disturbances under natural conditions in the cages, where they moved freely. Swelling and limping were classified as no change, mild and severe on the basis of severity[12] and inspection was done by a blinded inspector throughout the study.

Roentgenographic Examination Fourteen and 28 d after MIA injection, rats were checked with roentgenography to assess chronic morphological changes of the knee articular bones for narrowing, loss of joint region, and osteophyte formation.[13] Quantitative assessment was done by the veterinarian of Koryo Animal Clinic, Suwon, Korea.

Histopathological Analysis Twenty-eight days after MIA injection, rats (n=6) were anesthetized with diethyl ether and sacrificed. Their knee joints were removed and fixed in 10% neutral buffered formalin, decalcified with 10% formic acid, and embedded in paraffin. 5-μm sections were stained with hematoxylin & eosin (H&E) or safranin-O fast green (SOFG) and observed. Histopathological changes in each animal were quantitatively expressed by three grades for each finding.[14] Grading was done under the authority of Medplan Pathology Laboratories, Seoul, Korea.

Gelatinase Assays Rat articular cartilage samples of MIA-induced OA were harvested 2 (n=6) and 28 (n=6) days after MIA injection. Cartilages were snap frozen in liquid nitrogen and stored at −70 °C until assay. In assay, they were pulverized in 2 ml of homogenization buffer (pH 7.4), which consists of 50 mM Tris–HCl, 300 mM KCl, and 2.5 mM $MgCl_2$, in liquid nitrogen using pestle and mortar. For the MG-63 cells, cells were incubated in 12-well culture plate (5×10^4 cells/ml) for 24 h. Various concentrations of HPE (1.8×10^{-3}, 1.8×10^{-2}, 1.8×10^{-1}, 3.6×10^{-1} ml/ml) were added with serum-free media in 5 wells for each concentrations and incubated further for 72 h. Then gelatinase activities were measured by the gelatin zymography method described by Dumond *et al.*[15] Proteins were extracted from pulverized cartilage tissues and electrophoresed on 10% zymogram precast gels. After electrophoresis, the gels were incubated in renaturation buffer containing 2.5% Triton X-100, at room temperature for 40 min. Then gels were washed with distilled water and stored overnight at 37 °C in development solution. After developing, the gels were stained with 0.5% Coomassie brilliant blue R-250 staining solution in 40% methanol for 45 min and then destained in destaining solution containing 10% acetic acid. The cleared gels were captured and the area of each band was quantified with densitometric scanning analysis program (Science Lab 98 Image Gauge, version 3.12, Fuji Photo Film Co., Ltd., Tokyo, Japan).

Statistics All results are presented as the mean±S.E.M. The overall significance of the experimental results was examined by two-way analysis of variance. Differences between groups were considered significant at $p<0.05$ with the appropriate Bonferroni correction for multiple comparisons.

RESULTS

***In Vitro* Study. Gelatinase Assay** Compared to control group, HPE 1.8×10^{-1} ml/ml treated group showed significant inhibition of pro and active forms of MMP-2 as 61.6% and 44.8% of control, respectively. In the 3.6×10^{-1} ml/ml HPE treated group, the activities of pro and active forms of MMP-2 decreased to 60.2% and 24.3% lower than the control level (Fig. 1).

Cartilage Glycosaminoglycan Release In control, the level of GAG in the culture medium remained constant at approximately 1.5 μg/mg cartilage throughout the experiment. In the rhIL-1α-treated group, on the other hand, the level of GAG in culture medium dramatically increased at 48 h to 2.5 times the control value and persisted until the end of the

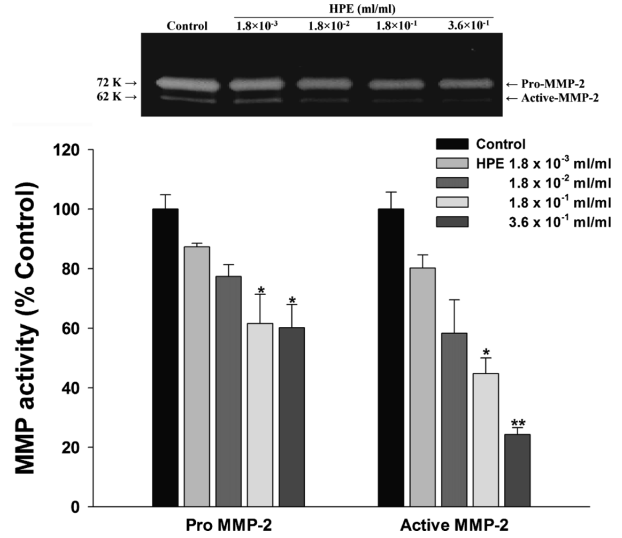

Fig. 1. Activity of MMP-2 (Gelatinase A) Assessed by Zymography in MG-63 Cells

Cells were incubated in 12-well culture plate (5×10^4 cells/ml) for 24 h. Various concentrations of HPE (1.8×10^{-3}, 1.8×10^{-2}, 1.8×10^{-1}, 3.6×10^{-1} ml/ml) were added with serum-free media in 5 wells for each concentrations. Cells were incubated further for 72 h and harvested for assay. High concentrations of HPE (1.8×10^{-1}, 3.6×10^{-1} ml/ml) inhibited the pro and active forms of MMP-2 activity in osteoblastic MG-63 cells. Each value represents the mean±S.E.M. from 5 wells per group. *, ** Significantly different ($p<0.05$, $p<0.01$) from control.

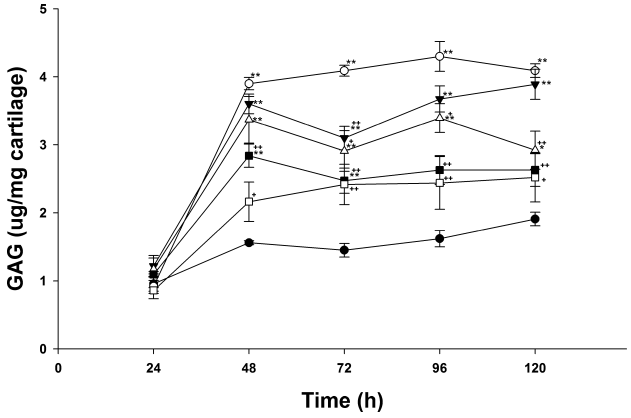

Fig. 2. GAG Release in Rabbit Articular Cartilage Explant Cultures at 24, 48, 72, 96, and 120 h

Rabbit articular cartilage explants were stimulated with rhIL-1α (5 ng/ml). The amount of GAG release stimulated by rhIL-1α (○) increased approximately 2.5-times compared to control (●) from 48 to 120 h. High concentrations of HPE [1.8×10^{-1} (△) and 3.6×10^{-1} (■) ml/ml media] and diclofenac [30 μM (□)] efficiently inhibited the GAG release. However, a moderate concentration of HPE [1.8×10^{-2} (▼) ml/ml media] slightly inhibited it. Each value represents the mean±S.E.M. from 5 articular cartilage explants cultures per group. *, ** Significantly different ($p<0.05$, $p<0.01$) from control. +, ++ Significantly different ($p<0.05$, $p<0.01$) from rhIL-1α.

experiment. HPE treatments attenuated the elevation in GAG release at all time points (Fig. 2).

***In Vivo* Study. Gross Observations** In the MIA-injected group, swelling and limping were first noted at 7 d. They subsided transiently and then reappeared at 14 d after intra-articular injection of MIA, after which they were gradually aggravated at 21 d (data not shown) and were the most severe at 28 d. At 28 d, swelling was attenuated by all doses of HPE while limping was reduced by 0.2 and 0.4 ml/kg HPE (Table 1).

Roentgenographic Examination At 14 d after MIA injection, rats underwent the first roentgenographic examination. Their roentgenographic examinations revealed some degenerative changes, such as irregularity or osteophytes on the surface of the cartilage and spurs (data not shown). At 28 d, rats underwent the second roentgenographic examination. Morphological changes were more apparent, showing rough edges of cartilage, bone lysis, and the tendency of patellar displacement. These changes were attenuated by HPE treatment (Figs. 3A—D, Table 2).

Table 1. Quantitative Summary of Gross Observations in MIA-Induced Osteoarthritic Rats Treated with HPE

Observatory changes (%)	Control	MIA					
		Vehicle	HPE (ml/kg)				Diclofenac
			0.022	0.066	0.2	0.4	3 mg/kg
Swelling							
No change	100	7	10	4	11	18	30
Mild	0	43	62	69	72	73	65
Severe	0	50	28	27	17	9	5
Limping							
No change	100	9	9	0	34	40	55
Mild	0	62	27	58	33	53	42
Severe	0	29	64	42	33	7	3

HPE or diclofenac was treated daily for 14 d after 2 weeks of OA induction by intraarticular injection of MIA. Scores are the percentage values of individual 12—16 rats per group.

Histopathological Analysis In the MIA-injected group, the joint cartilage had an irregular surface accompanied by ulceration and fibrillation. Exposure of subchondral bone and osteophytes were observed in both H & E- and SOFG-stained samples. Also, chondrocytes of joint cartilage were pale-stained, hypertrophied and disorganized. SOFG staining revealed clearly diffused PG depletion in joint cartilage tissues of MIA-injected rats. However, cartilage damage and PG loss were attenuated by HPE treatment (Figs. 3E—L). Summation of all pathologic finding scores in vehicle-treated MIA group and in 0.2 and 0.4 ml/kg HPE-treated MIA groups were 23.9±1.8, 14.9±1.7, and 10.9±1.7, respectively (Table 3).

Gelatinase Assay At 2 d after MIA injection, the activities of the pro and active forms of MMP-2 increased to 2.0- and 2.5-times that in the control group, respectively. Similarly, the activities of the pro and active forms of MMP-9 increased to 2.4- and 2.9-fold higher than the control level, respectively. All of the activities of the pro and active forms of MMP-2 and MMP-9 were significantly lowered by 0.4 ml/kg HPE. At 28 d after MIA injection, the activities of the pro and active forms of MMP-2 increased to 3.5- and 3.4-times those in the control group, respectively. On the other hand, the pro and active forms of MMP-9 exhibited activities increased by 1.8- and 1.6-times, which were lower than the activities at 2 d post-MIA injection. 0.4 ml/kg HPE treatment significantly lowered both MMP-2 and MMP-9 activities, but 0.2 ml/kg HPE treatment only lowered pro-MMP-2 activity (Fig. 4).

DISCUSSION

MIA not only inhibits connective tissue PG synthesis but also decreases ATP volume in cell cultures or incubated car-

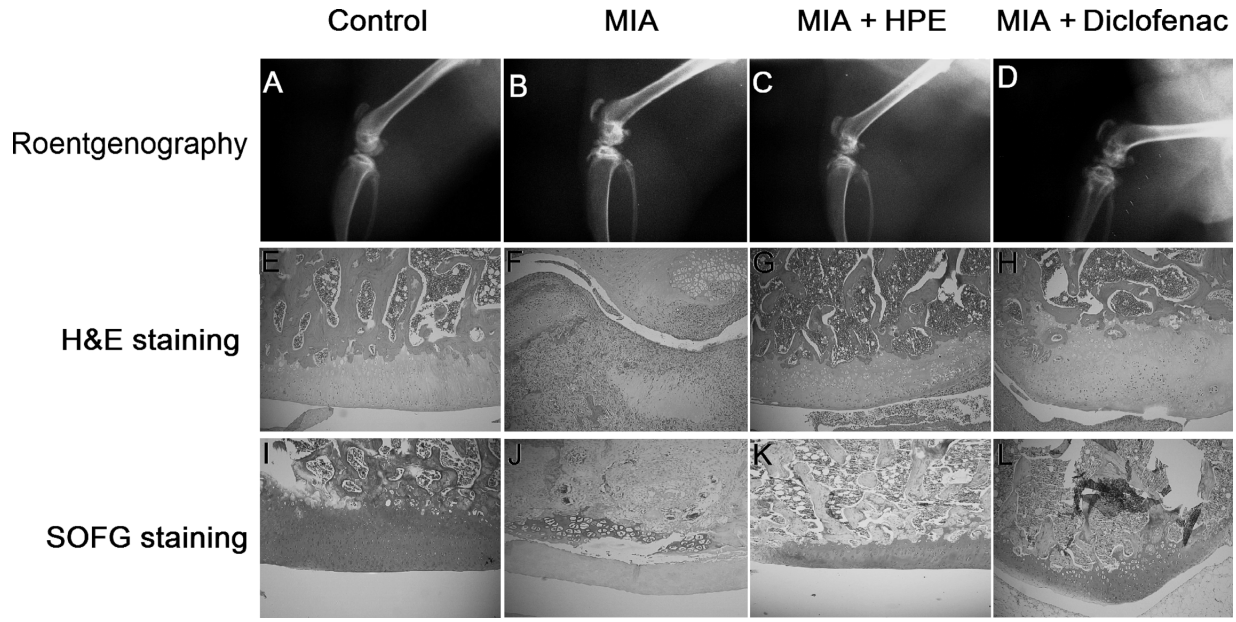

Fig. 3. Roentgenography and Histopathological Features of Osteoarthritic Lesions in the Knee Joint of Rats 28 d after Intra-articular Injection of MIA (A—D: Roentgenography; E—H: H & E Staining×100; I—L: SOFG Staining×100)

In roentgenography of knee joint, control (A) represents intact normal joint feature. Vehicle-treated MIA (B) shows a severely damaged joint with rough edges around the tibia and femur, indicative of bone lysis, swelling and tendency of patellar displacement. However, these damages were reduced significantly by treatment with 0.4 ml/kg HPE (C) and 3 mg/kg diclofenac (D). H & E stained control (E) represents the normal status of joint cartilage, whereas vehicle-treated MIA (F) represents severely damaged cartilage showing widespread cell necrosis, inflammation and loss of cartilage. However, treatment with 0.4 ml/kg HPE (G) and 3 mg/kg diclofenac (H) significantly reduced joint cartilage damage. In SOFG-stained samples, control (I) represents normal cartilage PG staining, whereas vehicle-treated MIA (J) represents severely damaged cartilage showing marked fibrillation and the depletion of safranin-O staining with separation of cartilage from subchondral bone. 0.4 ml/kg HPE (K) and 36 mg/kg diclofenac (L) treatments significantly reduced cartilage damage.

Table 2. Quantitative Summary of Roentgenographic Examination in MIA-Induced Osteoarthritic Rats Treated with HPE

Roentgenic changes (%)	Control	MIA					
		Vehicle	HPE (ml/kg)				Diclofenac
			0.022	0.066	0.2	0.4	3 mg/kg
Normal	88	7	0	0	0	0	0
Mild	12	36	67	75	83	93	90
Severe	0	57	33	25	17	7	10

HPE or diclofenac was treated daily for 14 d after 2 weeks of OA induction by intraarticular injection of MIA. Scores are the percentage values of individual 12—16 rats per group.

tilage tissues.[16] The injection of MIA into the knees of rats provides a model in which lesions resembling some aspects of human OA are produced quickly and has been suggested as a model for the study of chondroprotective drugs.[17] The rapid development of joint damage in the rat MIA model has advantages over spontaneous or surgical models in larger animals as smaller amounts of inhibitor and shorter time periods are required.

In the present study, we also investigated the effect of HPE on clinical and behavioral changes associated with MIA-induced OA. Swelling and limping were apparent as early as 7 d post-MIA injection, after which they transiently subsided. At 14 d, there was a second period of knee joint swelling and limping that was progressively aggravated until day 28. Previous work by Guzman and colleagues[18] showed that the majority of damage to articular tissue in the first 7 d after MIA injection was to the cartilage; bone damage was not typically present until after this period. The late onset of the second phase of pain behavior suggest that the efficacy of potential therapeutic agents in this model would clinically be most relevant when studied at later time points, such as 21 d after MIA injection. The efficacy of HPE on clinical and behavioral changes was not significant when administered acutely (data not shown). However, administration of HPE once daily for 14 d significantly reduced the severity of swelling and limping. These results suggested that HPE may have potential as a treatment for OA. Roentgenographic and histological observations strongly supported the behavior changes following MIA injection, as well as the protective effect of HPE. At 14 d, damage on joint area, such as osteophyte formation and roughening of bone surface in roentgenography, was noted in MIA-injected animals (data not shown). At 28 d after MIA injection, joint cartilage damage with massive roughening of bone surface and loss of cartilage was observed. Also, histological findings showed significant structural and cellular disorientations. HPE had some beneficial effects on these changes of articular cartilage and cells.

Measuring the amount of GAG released from cartilage tissue is a well known method for determining arthritic damage *in vitro* and has been practiced in various studies.[19] Cartilage comprises an extracellular matrix consisting of PGs, collagens (types II, IX, XI, and others), and water. Cartilage PGs consist of a protein core with GAG side chains.[20] As cartilage is damaged by inflammatory mediators such as rhIL-1α, PGs degrade and consequently release GAG. In our study, the amount of GAG released into culture medium significantly increased with rhIL-1α treatment, representing PG degradation and cartilage destruction. HPE did not inhibit GAG release at low to moderate concentrations but showed an inhibitory effect at high concentrations. This analysis

Table 3. Summary of Microscopic Findings

		MIA				
		Vehicle	HPE (ml/kg)			Diclofenac
			0.022	0.066	0.2	0.4	3 mg/kg
Structural changes in the joint							
Surface irregularities	+	0/4	1/4	1/4	3/4	2/4	2/4
	++	0/4	0/4	0/4	0/4	1/4	2/4
	+++	4/4	3/4	3/4	1/4	1/4	0/4
Average pathology score		3	2.5	2.5	1.5	1.2	1.5
Ulceration	+	1/4	2/4	0/4	1/4	2/4	1/4
	++	0/4	2/4	1/4	2/4	2/4	2/4
	+++	3/4	0/4	3/4	1/4	0/4	1/4
Average pathology score		2.5	1.5	2.8	2	1.5	2
Fibrillation of cartilage surface	+	0/4	2/4	0/4	1/4	2/4	3/4
	++	4/4	0/4	1/4	3/4	2/4	1/4
	+++	0/4	2/4	3/4	0/4	0/4	0/4
Average pathology score		2	2	2.8	1.8	1.5	1.3
Disorganization of chondrocytes	+	0/4	1/4	1/4	2/4	3/4	3/4
	++	3/4	3/4	3/4	2/4	1/4	0/4
	+++	1/4	0/4	0/4	0/4	0/4	1/4
Average pathology score		2.3	1.8	1.8	1.5	1.3	1.5
Exposure of subchondral bone	+	3/4	0/4	0/4	0/4	0/4	0/4
	++	0/4	0/4	1/4	0/4	0/4	0/4
	+++	1/4	0/4	1/4	0/4	0/4	0/4
Average pathology score		1.5	0	1.3	0	0	0
Cellular changes of chondrocyte							
Hypertrophy	+	0/4	1/4	1/4	2/4	1/4	0/4
	++	1/4	2/4	3/4	2/4	3/4	4/4
	+++	3/4	1/4	0/4	0/4	0/4	0/4
Average pathology score		2.8	2	1.8	1.5	1.8	2
Degeneration/Necrosis	+	0/4	0/4	1/4	1/4	4/4	2/4
	++	0/4	1/4	0/4	1/4	0/4	2/4
	+++	4/4	3/4	3/4	2/4	0/4	0/4
Average pathology score		3	2.8	2.5	2.3	1	1.5
Inflammatry cell infiltration	+	1/4	3/4	2/4	3/4	2/4	3/4
in synovial tissue	++	2/4	0/4	1/4	1/4	1/4	0/4
	+++	1/4	1/4	1/4	0/4	0/4	1/4
Average pathology score		2	1.5	1.8	1.3	1	1.5
Synovial cell proliferation	+	1/4	3/4	4/4	3/4	3/4	3/4
	++	3/4	1/4	0/4	0/4	1/4	1/4
	+++	0/4	0/4	0/4	1/4	0/4	0/4
Average pathology score		1.8	1.3	1	1.5	1.3	1.3
Safranin-O staining							
Reduction of staining in cartilage	+	0/4	0/4	1/4	3/4	1/4	1/4
	++	0/4	1/4	0/4	0/4	0/4	1/4
	+++	4/4	3/4	3/4	1/4	0/4	0/4
Average pathology score		3	2.8	2.5	1.5	0.3	0.8
Total pathology score (average±S.E.M.)		23.9±1.8	18.2±3.2	20.8±1.6	14.9±1.7*	10.9±1.7**	13.4±2.2**

+: Mild, ++: moderate, +++: severe. *, ** Significantly different ($p<0.05$, $p<0.01$) from vehicle-treated MIA.

reflected the histochemical appearance of the cartilage. For example, MIA injection induced the loss of cartilage PG as measured by SOFG staining. PG loss was followed by severe thinning of the cartilage and the development of lesions in the subchondral bone, which were attenuated by HPE treatment. Our data suggest that HPE may protect articular cartilage from degradation.

MMPs are a family of proteinases that together can degrade all extracellular matrix components. Type IV collagenases (gelatinases) are members of the family of MMPs and are thought to play an important role in the degradation of extracellular components. The gelatinase subclass can be divided into gelatinase-A (MMP-2) and gelatinase-B (MMP-9), which are capable of degrading types IV and V collagens,

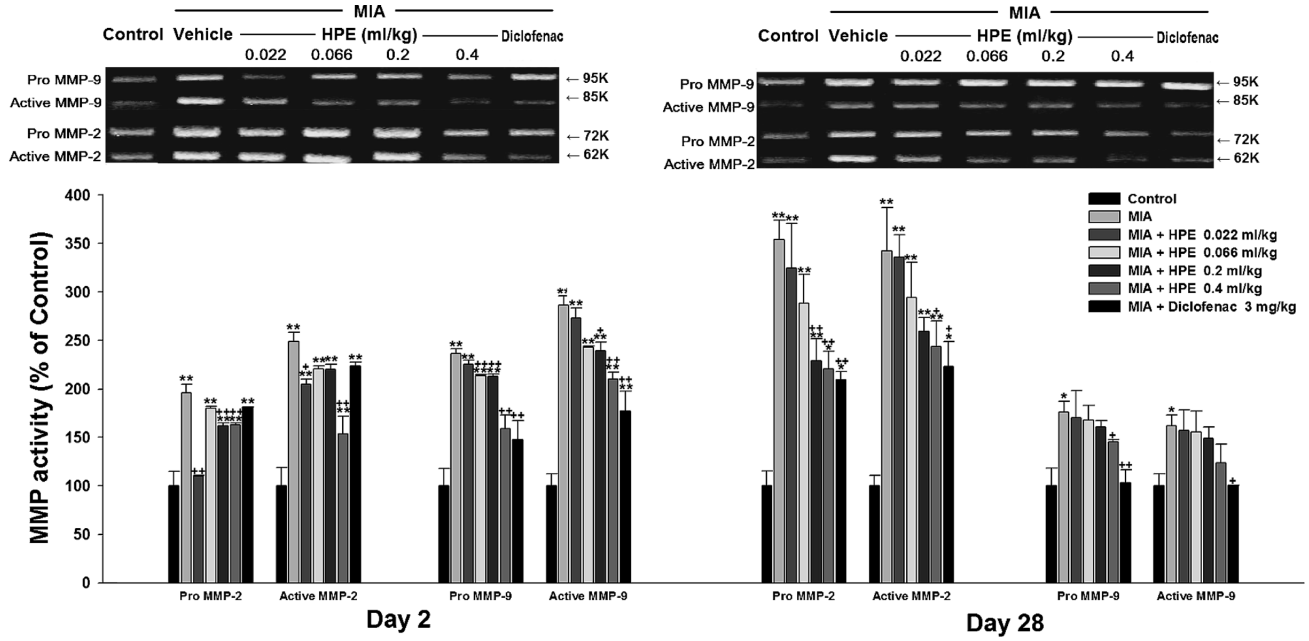

Fig. 4. Activities of the Pro and Active Forms of MMP-2 (Gelatinase A) and MMP-9 (Gelatinase B) Assessed by Zymography in Knee Joint Cartilages Obtained 2 and 28 d after MIA Injection

At 2 and 28 d after MIA injection, all of the activities of pro and active forms of MMP-2 and MMP-9 were significantly lowered by 0.4 ml/kg HPE. Each value represents the mean±S.E.M. from 6 rats per group. *, ** Significantly different ($p<0.05$, $p<0.01$) from control. +, ++ Significantly different ($p<0.05$, $p<0.01$) from vehicle-treated MIA.

elastin and gelatin.[21] These MMPs are secreted as latent precursors and can be activated by limited proteolysis, which results in a loss of about 10 kDa of molecular weight. The increased expression of MMP-2 and -9 in the synovium of patients with arthritic effusions superiorly reflects the inflammatory condition of the joints, and a positive correlation between MMP-9 production and rapid destruction of the hip joint has been described in OA.[22] The zymographic profile and MMP expression can act as a useful diagnostic adjunct in patients with OA, providing precise information on the condition of the articular cartilage and the breakdown of its matrix. In the present study, high concentrations of HPE significantly lowered MMP-2 activity in osteoblastic cell line which is known to produce MMP-2 in normal condition.[23] This shows the possibility of HPE as an overexpressed-MMPs inhibitor in OA. In MIA-injected rats, MMP-2 activity was significantly increased after the first 2 d and further increased at 28 d. MMP-2 activity at 2 d was suppressed by HPE, but not by diclofenac, and at 28 d by both HPE and diclofenac. It is interesting to note that MMP-9 activity increased at 2 d after MIA injection with a subsequent decline in activity at 28 d. The administration of HPE for the initial 2 d after MIA injection was efficacious. There is strong circumstantial evidence that MMP-2 participates in the turnover of normal cartilage matrix, whereas MMP-9 and some MMP-2 facilitate the progressive destruction of cartilage matrix in OA.[24] Our results indicate that HPE inhibits collagen degradation through inhibition of MMP-2 and -9 in the early stage of OA.

Taken together, our results demonstrate that HPE might reduce cartilage degradation through suppressing the expression of MMPs. Because HPE can be administered over long periods of time without any known serious side effects, its possible role as a therapeutic in OA deserves consideration. In addition, the precise mechanisms involved in its effect on MMP expression and cartilage protection need to be further investigated.

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REFERENCES

1) Park K. C., Park E. J., Kim E. R., Kim Y., Chung S. H., Cho B. W., Kim S., Jin M., *Biochem. Biophys. Res. Commun.*, **331**, 1469—1477 (2005).
2) Poitras S., Avouac J., Rossignol M., Avouac B., Cedraschi C., Nordin M., Rousseaux C., Rozenberg S., Savarieau B., Thoumie P., Valat J. P., Vignon E., Hilliquin P., *Arthritis Res. Ther.*, **9**, R126 (2007).
3) Tonello G., Daglio M., Zaccarelli N., Sottofattori E., Mazzei M., Balbi A., *J. Pharm. Biomed. Anal.*, **14**, 1555—1560 (1996).
4) Hooper K. C., *J. Physiol.*, **148**, 283—290 (1959).
5) Uszynski M., *Thromb. Res.*, **16**, 689—694 (1979).
6) Mochizuki H., Kada T., *Mutat. Res.*, **95**, 457—474 (1982).
7) Sabapatha A., Gercel-Taylor C., Taylor D. D., *Am. J. Reprod. Immunol.*, **56**, 345—355 (2006).
8) Hong H. T., Kim H. J., Lee T. K., Kim D. W., Kim H. M., Choo Y. K., Park Y. G., Lee Y. C., Kim C. H., *J. Ethnopharmacol.*, **79**, 143—148 (2002).
9) Yeom M. J., Lee H. C., Kim G. H., Shim I., Lee H. J., Hahm D. H., *Biol. Pharm. Bull.*, **26**, 1472—1477 (2003).
10) Sandy J. D., Brown H. L., Lowther D. A., *Biochim. Biophys. Acta*, **543**, 536—544 (1978).
11) Bove S. E., Calcaterra S. L., Brooker R. M., Huber C. M., Guzman R. E., Juneau P. L., Schrier D. J., Kilgore K. S., *Osteoarthritis Cartilage*, **11**, 821—830 (2003).
12) Choi J. H., Kim D. Y., Yoon J. H., Youn H. Y., Yi J. B., Rhee H. I., Ryu K. H., Jung K., Han C. K., Kwak W. J., Cho Y. B., *Osteoarthritis Cartilage*, **10**, 471—478 (2002).
13) Sakano Y., Terada N., Ueda H., Fujii Y., Hamada Y., Akamatsu N., Ohno S., *Med. Electron. Microsc.*, **33**, 246—257 (2000).
14) Kobayashi K., Imaizumi R., Sumichika H., Tanaka H., Goda M.,

Fukunari A., Komatsu H., *J. Vet. Med. Sci.*, **65**, 1195—1199 (2003).
15) Dumond H., Presle N., Pottie P., Pacquelet S., Terlain B., Netter P., Gepstein A., Livne E., Jouzeau J. Y., *Osteoarthritis Cartilage*, **12**, 284—295 (2004).
16) Mason R. M., Spencer C. A., Palmer N. T., "Chondrocyte Energy Metabolism and Its Modulation by Iodoacetate and Diclofenac," Hogrefe and Huber, Toronto, 1989.
17) Barve R. A., Minnerly J. C., Weiss D. J., Meyer D. M., Aguiar D. J., Sullivan P. M., Weinrich S. L., Head R. D., *Osteoarthritis Cartilage*, **15**, 1190—1198 (2007).
18) Guzman R. E., Evans M. G., Bove S., Morenko B., Kilgore K., *Toxicol. Pathol.*, **31**, 619—624 (2003).
19) Stevens A. L., Wheeler C. A., Tannenbaum S. R., Grodzinsky A. J., *Osteoarthritis Cartilage*, **16**, 489—497 (2008).
20) Trelstad R., Kemp P., "Matrix Glycoproteins and Proteoglycans," WB Saunders, Philadelphia, 1993.
21) Mizui T., Ishimaru J., Miyamoto K., Kurita K., *Br. J. Oral. Maxillofac. Surg.*, **39**, 310—314 (2001).
22) Chu S. C., Yang S. F., Lue K. H., Hsieh Y. S., Wu C. L., Lu K. H., *Connect. Tissue Res.*, **45**, 142—150 (2004).
23) Meikle M. C., Bord S., Hembry R. M., Compston J., Croucher P. I., Reynolds J. J., *J. Cell Sci.*, **103**, 1093—1099 (1992).
24) Hsieh Y. S., Yang S. F., Chu S. C., Chen P. N., Chou M. C., Hsu M. C., Lu K. H., *Arthroscopy*, **20**, 482—488 (2004).