Effect of Mixed Extract of *Panax Notoginseng*, *Rehmanniae Radix* and *Acanthopanax Cortex* (AIF) on Experimentally Induced Osteoarthritis

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Abstract – The objective of the present study was to evaluate the effect of a mixed extract of three herbs, *Panax Notoginseng*, *Rehmanniae Radix* and *Acanthopanax cortex* (AIF), for the treatment of horses with experimentally induced osteoarthritis. Twelve healthy male horses were included in this study. Horses were assigned to one of two groups: the AIF group (n=6) or the control group (n=6). Osteoarthritis was induced in all horses by intraarticular injection of sodium monoiodoacetate (0.12 mg/kg). Horses in the AIF group received 3 g of AIF with food daily, and those in the control group received food only. Treatment began on the day of intraarticular injection. Clinical and radiographic evaluations were performed every 2 weeks. At week 12, horses were euthanatized, and postmortem gross pathologic and histologic examinations of the middle carpal joint were performed. There were no significant differences in clinical values between the two groups. Radiographic evaluation revealed that the percentages of narrowness of joint space width in the control group were significantly higher than those in the AIF group ($p < 0.02$). On gross pathologic examination, the mean total dimensions of articular cartilage erosions and fibrillations in the control group ($101.5 \pm 41.5 \text{ mm}^2$) were significantly wider than those in the AIF group ($29.3 \pm 39.7 \text{ mm}^2$; $p < 0.01$). On histopathologic evaluation, significantly higher grades of staining intensity and lower empty lacunae (EL) ratios were found in the AIF group ($p < 0.03$). The present study revealed that AIF had significant disease modifying effects in horses with experimentally induced osteoarthritis.

Keywords: *Panax notoginseng*, *Rehmanniae radix*, *Acanthopanax*, Horses, Osteoarthritis, Lameness

INTRODUCTION

Joint disease is the most common cause of lameness in horses and represents a major caseload for the equine clinician (Bolam *et al.*, 2006; Goodrich and Nixon, 2006). In mature horses, osteoarthritis is specifically important and results in considerable athletic and economic losses (Bolam *et al.*, 2006). Considered a disorder of movable joints, osteoarthritis is characterized by deterioration of articular cartilage and the formation of new bone at joint surfaces and margins (Van der Harst *et al.*, 2005).

The primary goals in developing drugs for equine osteo-

arthritis treatment include pain alleviation and inhibition of inflammation (Frisbie *et al.*, 2009c). The therapeutic goals are mainly achieved by use of nonsteroidal anti-inflammatory drugs (NSAIDs), corticosteroids, polysulfated glycosaminoglycans, and hyaluronan (Lee, 2003; Goodrich and Nixon *et al.*, 2006). Although various treatments are considered effective, some horses are refractory to standard treatment (Frisbie *et al.*, 2009a). NSAIDs, including phenylbutazone, flunixin meglumine, ketoprofen, naproxen, and carprofen, are some of the first treatments of choice for equine osteoarthritis, but in cases of horses with chronic pain who require long term therapy for maintaining their quality of life, NSAIDs must be used with caution because of the potential for toxic effects (Goodrich and Nixon, 2006).

The popularity of alternative treatment options, such as the use of herbs, for horses with cartilage inflammation is

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increasing (Pearson *et al.*, 2007). A previous study described the chondroprotective effect of a commercially available composite of five herbs, *Harpagophytum procumbens*, *Urtica dioica*, *Taraxacum officinale*, *Symphytum officinale*, and *Arctium lappa* (Mobility™)(Pearson *et al.*, 2007), in interleukin-1 stimulated cartilage. Avocado and soybean unsaponifiable extracts have also been reported to modify disease by reducing articular cartilage erosion and synovial hemorrhage and increasing the synthesis of glycosaminoglycans in horses with experimentally induced arthritis (Kawcak *et al.*, 2007). These studies suggest that herbal products may be useful as adjuncts in equine arthritis (Kawcak *et al.*, 2007; Pearson *et al.*, 2007).

Recently, the therapeutic effects of a water extract from three herbs, *Panax Notoginseng*, *Rehmanniae Radix* and *Acanthopanax cortex* (AIF), on osteoarthritis have been investigated (Chang *et al.*, 2005; Chang *et al.*, 2008; Park *et al.*, 2009). Previous studies have shown that AIF has a protective effect on the progression of arthritis in rat and mouse models (Chang *et al.*, 2005; Chang *et al.*, 2008). An in-vitro study proved that AIF has anti-inflammatory properties and protects cartilage by inhibiting the production of tumor necrosis factor- α (TNF- α), interleukin-1 (IL-1), inducible nitric oxid (iNO), and matrix metalloproteinase-13 (MMP-13; 10). A prospective placebo-controlled clinical trial on human patients with idiopathic osteoarthritis showed significant pain alleviation and improvements in stiffness and physical function in a group treated with AIF twice daily for 6 weeks compared to the control group. This study also showed no significant differences in the incidence of adverse events between the group treated with AIF and the control group (Park *et al.*, 2009).

Our hypothesis was that a mixed extract of three herbs, *Panax Notoginseng*, *Rehmanniae Radix* and *Acanthopanax cortex*, would have a therapeutic effect on equine osteoarthritis. The purpose of the present study was to evaluate the effect of AIF, a mixed extract of three herbs, on horses with experimentally induced osteoarthritis.

MATERIALS AND METHODS

Animals

12 healthy male Thoroughbred horses, aged 2 to 4 years, were included in this study. Horses were housed in stalls for two weeks for an acclimation period prior to the beginning of the experiment. Before initiation of the study, lameness evaluations, flexion tests and radiographics examination of the carpal joints were performed to verify that the horses did not have preexisting lameness or any radiographic examination indication of osteoarthritis in the mid-

dle carpal joints. Body condition, complete blood counts, and serum chemistry profiles were assessed to ensure that the horses were generally healthy. Horses were assigned to one of two groups, with 6 horses in each group: the control or the mixed extract of *Panax notoginseng*, *Rehmanniae Radix*, and *Acanthopanax cortex* (AIF) treatment group. This study protocol adhered to the strict guidelines in the "Guide for the Care and Use of Laboratory Animals" of Seoul National University (Seoul, South Korea).

Experimental induction of osteoarthritis

Sterile sodium monoiodoacetate (Sigma-Aldrich, Castle Hill, Australia) solution was prepared in a concentration of 100 mg/ml. Horses were sedated with intravenous administration of 0.01 mg/kg of detomidine (Dormosedan®, pfizer animal health, New York City, NY, USA). The left carpus was clipped and aseptically prepared. Intraarticular injection of sodium monoiodoacetate (0.12 mg/kg) was performed with a 5 ml syringe and 21-gauge needle in the semiflexed left middle carpal joints of the 12 standing horses. Successful entry into the joint was confirmed by aspiration of synovial fluid while pulling the plunger.

Treatment

Treatment began on the day of intraarticular injection. Horses in the AIF group received 3 g of mixed extracts of *Panax notoginseng*, *Rehmanniae Radix*, and *Acanthopanax cortex* (AIF: active ingredient of AIF-Equine®, Oscotec Inc and CTC BIO Inc, Cheonan and Hwasung, Choongnam and Gyeonggi, Korea) once daily with ordinary food (Pro-horse®, Cargill Agri Purina, Pyeongteak, Gyeonggi, Korea), and horses in the control group received food only.

Exercise

Horses were housed in stalls. Beginning 15 days after intraarticular injection according to previous studies (Frisbie *et al.*, 2009a, b), horses were lunged at a trot in a round yard for an hour, 5 days each week during the study period.

Clinical and radiographic evaluations

Clinical assessments, including a lameness test, palpation and flexion tests for signs of edema, and pain of the middle carpal joints, were performed prior to osteoarthritis induction (baseline) and every two weeks throughout the study. Lameness was graded with the horse trotting on a hard surface. All clinical outcomes were graded on a 0 to 3 scale (0 - normal; 1 - mild; 2 - moderate; 3 - severe). Presence of any adverse effects such as diarrhea and

weight loss was monitored throughout the study period. Radiographic evaluation of the left carpus was performed prior to osteoarthritis induction and every two weeks throughout the entire study period. Standard dorsopalmar and lateromedial views were obtained. The radiographs were performed while the horse was bearing weight evenly on all four limbs; at this time the limb would be vertically radiographed. Dorsopalmar views were obtained with x-ray beams aligned horizontally and the cassette held vertically at right angles to the beam. The x-ray beam was centered on the middle carpal joint. In order to obtain true dorsopalmar and lateromedial views, the beam was best oriented to the limb horizontally as possible (PXP-16HF, POSKOM Co., Ltd., Goyang, Korea). The images were viewed using the manufacturer's software (PhenixVision xViewer, PhenixVision Co., Ltd. Seongnam, Korea). Radiographic images were also evaluated for the presence of osteophytes, enthesophytes, or subcondral bone sclerosis. Finally, an investigator who was unaware of the treatment each horse received used an electronic caliper installed in the viewer program on dorsopalmar views to measure the widths of joint space between the distal radiocarpal bone and third carpal bone, and between the distal third carpal bone and third metacarpal bone, and the percentage of joint space narrowness was compared between two groups.

Postmortem evaluation of joints

At the termination of the study (12 weeks after intra-articular injection), all horses were euthanized, and gross pathologic examination of the articular cartilage of the middle carpal joints was performed. The middle carpal joints were disarticulated, pictures were taken with a ruler placed in the same plane as the cartilage surface, and the total dimensions of cartilage erosion and fibrillation were calculated using an UTHSCSA image tool software program (University of Texas Health Science Center, San Antonio, TX, USA).

For histopathologic evaluation, samples of articular cartilage (approximately $5 \times 7 \text{ mm}^2$) were harvested from the standardized site which is radial facet of the third carpal bone following gross pathologic examination. Samples were fixed in neutral buffered 10% formalin for 14 days, embedded in paraffin, sectioned into $4 \mu\text{m}$ slices, mounted on glass slides, and stained with Safranin O and hematoxylin and eosin (H&E). Digital photomicrographs were obtained, and the intensity of staining of three different areas of each sample was graded on a 0 to 4 scale (0 indicated no uptake, 1 indicated mild stain uptake in the territorial matrix, 2 indicated mild stain uptake in 75% or more of the

interterritorial matrix and complete stain uptake in the territorial matrix, 3 indicated moderate stain uptake in 75% or more of the interterritorial matrix and complete stain uptake in the territorial matrix, 4 indicated complete stain uptake in the territorial matrix and interterritorial matrix) and averaged by a blinded investigator in accordance with a previous study (Gustafson *et al.*, 1992). Territorial matrix was defined as the layers that surround chondrocytes in cartilage. Interterritorial matrix was defined as the matrix that occupies the majority of space between chondrocytes. Empty lacunae represented the lacunae that have no cells within the lacunar space. The proportion of empty lacunae vs the total number of lacunae (EL ratio = number of empty lacunae / counted 100 lacunae) in chondrocytes of the articular cartilage was compared between the control group and the AIF group.

Statistical analysis

Data was expressed as mean $\pm$ SD. Statistical analysis was performed with the SPSS 12.0k for Windows (SPSS Inc., Chicago, IL, USA). The Mann-Whitney U test was used to compare data on clinical outcomes, joint widths at baseline, total dimensions of cartilage erosion, grades for staining intensity, and EL ratios between the AIF group and control group. The Wilcoxon signed rank test was employed to compare data between joint widths at baseline and widths 12 weeks after osteoarthritis induction. Values of $p < 0.05$ were considered significant.

RESULTS

Clinical and radiographic evaluations

There were no significant differences in scores for pain, edema, and lameness between the two groups at any time point ($p > 0.05$) (Table I). No adverse effect was noted in either group.

No radiographic sign of osteophyte or enthesophyte formation, or subcondral bone sclerosis was observed in any group at any time point. There were no significant differences in the widths of joint space between the distal radiocarpal bones and third carpal bones ($p = 0.165$, $p > 0.05$), or between the distal third carpal bones and third metacarpal bones ($p = 0.160$, $p > 0.05$) before osteoarthritis induction (baseline). Significant narrowing of the joint space width between the distal radiocarpal bones and third carpal bones was detected 12 weeks after osteoarthritis induction when compared to baseline in both groups ($p < 0.04$) (Table II). There was significant narrowing of the joint space width between the distal third carpal bone and third metacarpal bone in the control group ($p = 0.03$), but no significant

Table I. Scores for clinical evaluations of horses in the control and in the mixed extract of *Panax notoginseng*, *Rehmanniae Radix*, and *Acanthopanax cortex* (AIF) treatment group. Scores are expressed as mean $\pm$ SD. Weeks represent weeks after induction of osteoarthritis. No statistical differences were found between the AIF and control groups at any time point

Weeks	Pain		Edema		Lameness	
	AIF	Control	AIF	Control	AIF	Control
0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0
2	0.0 $\pm$ 0.0	0.2 $\pm$ 0.4	1.3 $\pm$ 1.0	1.2 $\pm$ 0.8	1.7 $\pm$ 0.5	1.5 $\pm$ 1.4
4	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	1.3 $\pm$ 0.8	1.2 $\pm$ 0.8	1.0 $\pm$ 0.9	1.3 $\pm$ 1.2
6	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.8 $\pm$ 0.8	0.7 $\pm$ 0.5	0.2 $\pm$ 0.4	0.0 $\pm$ 0.0
8	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.5 $\pm$ 0.8	0.3 $\pm$ 0.5	0.0 $\pm$ 0.0	0.3 $\pm$ 0.5
10	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.2 $\pm$ 0.4	0.2 $\pm$ 0.4	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0
12	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.2 $\pm$ 0.4	0.2 $\pm$ 0.4	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0

Table II. Joint space widths between the distal radiocarpal bone and third carpal bone (RC-3C), between the distal third carpal bone and third metacarpal bone (3C-3MC), and the percentage of narrowness of the joint space width

	Group	Baseline (mm)	At 12 weeks (mm)	Narrowness (%)	<i>p</i> -value ^a
RC-3C	AIF	0.11 $\pm$ 0.01	0.09 $\pm$ 0.01 ^b	14.39 $\pm$ 7.97	0.004
	Control	0.11 $\pm$ 0.01	0.08 $\pm$ 0.01 ^b	39.32 $\pm$ 7.50	
3C-3MC	AIF	0.09 $\pm$ 0.01	0.08 $\pm$ 0.01	7.04 $\pm$ 8.36	0.015
	Control	0.09 $\pm$ 0.01	0.07 $\pm$ 0.01 ^b	27.31 $\pm$ 10.62	

Values are expressed as mean $\pm$ SD. ^aIndicates *p*-values when percentages of narrowness are compared between the AIF group and the control group. ^bIndicates values had significant differences ($p < 0.05$) when compared to baseline.

Fig. 1. Radiographic features of the left middle carpal and carpometacarpal joints in the AIF (A) Joint before treatment, (B) Joint 12 weeks after osteoarthritis induction) and control groups (C) Joint before treatment, (D) Joint 12 weeks after osteoarthritis induction). The degree of joint space width narrowing is more severe in the control group (D) compared to that in the AIF group (B) on the images 12 weeks after osteoarthritis induction.

Fig. 2. Articular cartilage erosions and fibrillations (black arrows) in the left middle carpal joints of a horse in the AIF group (A, C) and in the control group (B, D). Total number and dimension of lesions seen in the control group (n=2, 35.94 mm²) are remarkably higher than those in the AIF group (n=8, 148.22 mm²). Red arrow indicates artifact. A square with dotted lines in C indicates sampling site for histopathological evaluation.

Table III. Grades of staining intensity and empty lacunae to total lacunae ratios (EL ratios) found via Safranin O and H&E staining of the articular cartilage of the third carpal bone

		AIF	Control	<i>p</i> -value
Safranin O	Grade	2.7 ± 1.4	0.3 ± 0.5	0.009
	EL ratio	5.4 ± 1.5	8.9 ± 2.8	0.025
H&E	Grade	2.2 ± 1.2	0.5 ± 0.8	0.026
	EL ratio	5.0 ± 2.4	9.6 ± 2.1	0.015

Values are expressed as mean ± SD.

narrowing was observed in the AIF group ($p=0.10$, $p>0.05$; Table II). The percentages of narrowness of joint space width between the distal radiocarpal bone and third carpal bone and between the distal third carpal bone and third metacarpal bone in the control group were significantly higher than those in the AIF group ($p<0.02$)(Table II, Fig. 1).

Gross and histopathological evaluations

At necropsy, distension or thickening of joint capsule was not observed in any group. The mean total dimensions of articular cartilage erosions and fibrillations in the control group ($101.5 \pm 41.5 \text{ mm}^2$) were significantly higher than those in the AIF group ($29.3 \pm 39.7 \text{ mm}^2$, $p<0.01$)(Fig. 2).

Histologic evaluation of the articular cartilage via staining of Safranin O and H&E revealed significantly higher grades of staining intensity and significantly lower EL ratios in the AIF group versus control group ($p<0.03$)(Table III, Fig. 3).

No specific signs of inflammation or fibrosis were found

Fig. 3. Digital photomicrographs of sections of articular cartilage from the third carpal bone of a horse in the AIF group (A, B) and in the control group (C, D). Both Safranin O and H&E staining demonstrate higher staining intensity in the AIF group. Bar: 100 μm .

around the synovial membrane in either group.

DISCUSSION

The present study revealed that administration of a mixed extract of *Panax notoginseng*, *Rehmanniae Radix*, and *Acanthopanax cortex* (AIF) significantly reduced the radiological, gross and histopathological changes caused by osteoarthritis in horses with experimentally induced osteoarthritis. These findings suggest that AIF may have chondroprotective effects in equine osteoarthritis.

The three herbs used in this study have been widely used as anti-inflammatory and anti-pyretic drugs in human herbal medicine in eastern Asia for over 2000 years, and their therapeutic dosages and safety have been demonstrated (Park *et al.*, 2009). *Panax notoginseng* stimulates blood flow, has hematopoietic, anti-inflammatory, and analgesic effects, and has been used for the treatment of cardiovascular diseases and contusions (Li and Chu, 1999; Yoshikawa *et al.*, 2001; Chang *et al.*, 2005; Chang *et al.*, 2007). *Rehmanniae Radix* improves hematopoiesis and the secretion of endocrine glands, alleviates arthritis symptoms, and inhibits the formation of osteoclasts (Oh *et al.*, 2003). *Acanthopanax cortex* has anti-inflammatory and immunomodulatory effects, has been used to improve energy, and alleviates symptoms of arthritis (Wang *et al.*, 1991; Wang *et al.*, 1992; Chang *et al.*, 2005). Previous studies have revealed that the mixed extract of these three herbs (AIF) synergistically inhibits the inflammatory process and cartilage destruction, and has disease modifying effects in mouse and rat osteoarthritis models (Chang *et al.*, 2005; Chang *et al.*, 2008). A clinical trial demonstrated that AIF has symptom modifying effects without causing any adverse effects in human patients (Park *et al.*, 2009). The present study demonstrated that, in an experimentally induced osteoarthritis model in horses, AIF has a disease modifying effect and no apparent association with adverse events.

In this study, osteoarthritis was induced by intraarticular injection of sodium monoiodoacetate. Sodium monoiodoacetate has been used in horses for the experimental induction of cartilage degeneration at low doses (range 0.12 to 0.16 mg/kg; 13) and for chemical arthrodesis at high doses (range 50 to 400 mg per horse; 20) (Dowling *et al.*, 2004). Sodium monoiodoacetate causes dose-dependent cartilage degeneration that is characterized by fibrillation of cartilage and depletion of glycosaminoglycans and proteoglycans (Gustafson *et al.*, 1992). In the present study, fibrillation and erosion of cartilage were observed on postmortem gross pathology examination, and histopatho-

logic evaluation showed low Safranin O staining, indicating depletion of glycosaminoglycans and proteoglycans. These results demonstrate that osteoarthritis was adequately induced by the intraarticular injection of sodium monoiodoacetate in this study.

This study did not show any significant differences in clinical values between the AIF and control groups; this result is in contrast to results of a previous clinical trial performed in human patients in which the same treatment was used (Park *et al.*, 2009). One possible explanation for the discrepancy is the difference in species of study subjects; there are limitations to detecting pain in horses compared to humans. Another difference is that the human study was performed in patients with spontaneous osteoarthritis, and this study used an experimental model (Park *et al.*, 2009). Frequency and period of the treatment were also different between the two studies; this study gave AIF once daily for 12 weeks, while the human study administered AIF twice daily for 6 weeks (Park *et al.*, 2009). AIF will need to be studied in horses with spontaneous osteoarthritis using various administration protocols to determine its effectiveness.

Radiographic evaluation revealed significant decreases in the percentages of narrowness of the joint space widths in the AIF group compared to the control group. Gustafson *et al.* (1992) explained that joint space narrowing after intraarticular injection of sodium monoiodoacetate may reflect loss of compressive stiffness in cartilage due to depletion of proteoglycans. Therefore, our radiographic results suggest that, in horses with chemically induced osteoarthritis, AIF may protect cartilage from the depletion of proteoglycans. Results of gross and histopathologic findings also support the suggestion by showing consistent data; gross pathologic examination indicated significantly less articular cartilage erosions and fibrillations in the AIF group compared to control. In addition, histopathologic evaluation revealed that, compared to control, the AIF group showed a significantly higher staining of Safranin O and significantly lower EL ratio. The decreased cartilage destruction in the AIF group that was verified by gross and histopathologic evaluation paralleled findings in previous studies, which were performed in mouse and rat models (Chang *et al.*, 2005; Chang *et al.*, 2008). Features of synovitis were not found in gross and histopathologic examination in either group. A prior study reported that microscopic changes in the synovial membrane were not observed when subjects were given a low dose (0.09 mg/kg) of intraarticular sodium monoiodoacetate, and higher doses tended to induce more severe damage (Gustafson *et al.*, 1992). Authors of the study stated that, if the synovial

membrane was disrupted at a low dose, 12 weeks of the study period may have allowed regeneration of the membrane (Gustafson *et al.*, 1992). Therefore, a higher dose of sodium monoiodoacetate might be useful in order to determine the effect of treatment on synovitis in horses.

There are several limitations of this study. The number of horses in each group was relatively small, and the data have large standard deviations. Clinical evaluations were performed by persons who were aware of the treatment, and there was no significant difference in any clinical values between groups. Proteoglycans in articular cartilage matrix were not directly evaluated. Despite its blunt detection rate, the Safranin 'O' staining method was used for histopathologic evaluation. Thus, if a more accurate outcome is required, immunohistochemistry with monoclonal antibody is recommended.

In this study, AIF had significant disease modifying effects at gross and histopathological levels in horses with experimentally induced osteoarthritis. AIF could serve as an adjunctive therapy to suppress the progression of cartilage destruction in horses with osteoarthritis. Long term clinical trials in horses with spontaneous osteoarthritis would be needed to determine if AIF has symptom modifying effects.

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