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Contributed Paper

Therapeutic Effects of Short-term Supplementation of 0.5 and 1.0% Pineapple Shell on Rabbits with Experimentally Induced Osteoarthritis

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ABSTRACT

Treatment of osteoarthritis using bromelain has been widely utilized; however, the cost of bromelain is expensive because high technology is required for its extraction from pineapple. Therefore, in the present study dry pineapple shell at 0.5 and 1.0% was exploited as a dietary supplement for rabbits with experimentally induced osteoarthritis. Gene and protein expression, biomarker levels and histopathological scores were used to evaluate the effect of pineapple shell on osteoarthritis treatment. The results showed no significant difference ($P>0.05$) in the level of biomarkers and histopathological scores among three groups (non-treated control, and daily oral administration of 0.5 and 1.0% dry pineapple shell). The relative expressions of TIMP-1, MMP-1 and MMP-3 were significantly different between the 1.0% dry pineapple shell group and the control group. Daily oral administration of 1.0% pineapple shell for 3 months could alter the expression of anabolic genes; but this level was not sufficient for improving biomarker levels and histological scores. Supplementation with higher dosages and for longer periods of time should be considered before performing future clinical trials.

Keywords: pineapple shell, gene expression, protein expression

1. INTRODUCTION

Osteoarthritis (OA) is one of the most prevalent diseases of the joints. It is characterized by degeneration of articular cartilage, loss of chondrocytes, and impaired joint function; this is the result of many complex mechanisms such as erosion of articular cartilage, degradation of proteoglycan (PG), and collagen network disruption [1]. Until recently, the only therapies available to patients with OA were

short-term relief agents, chondroprotective agents [2,3], proliferative or regenerative therapies [4], or total surgical replacement of articular cartilage [5]. However, OA animals and their respective owners would prefer treatments that are cost-effective with fewer side effects. Therefore, oral supplements are of great interest.

Plant extracts and phytotherapeutic drugs have been used as alternative treatments [6]. Bromelain, which is extracted from pineapple (*Ananas comosus*), contains a number of proteolytic enzymes that are considered to have a range of beneficial properties, such as anti-inflammatory, analgesic, anti-oedematous, antithrombotic, and fibrinolytic effects [7]. A number of clinical trials have assessed the use of bromelain in treatment of joint inflammation [8,9]. These have also included open studies or equivalent studies designed to assess the comparative effectiveness against standard NSAID treatment [10]. Their findings suggest that bromelain may be beneficial in the treatment of OA, and could be equally as effective as standard NSAID treatment. A previous report from our group [11] found that bromelain can decrease the apoptotic rate of canine chondrocytes. It also significantly activated the proliferation of those cells, while not adversely affecting their viability. Furthermore, the results showed significantly decreased TIMP-1 and MMP-3 mRNA relative expression in bromelain-treated groups.

Many dietary supplements from natural products, such as devil's claw (*Harpagophytum procumbens*) [12], turmeric (*Curcuma longa*) [13] and ginger (*Zingiber officinale*) [14], are readily available and have been approved for OA treatment. We hypothesized that bromelain from pineapple shell could be used as a dietary supplement in order to reduce the progression of OA. So this preliminary study was designed to investigate the potential of pineapple shell supplement to alleviate clinical signs of OA stifle joint disease in rabbits over a three-month period.

2. MATERIALS AND METHODS

2.1 Experimental Animals

Thirty-two one-year-old female rabbits (95% New Zealand), body weight 2.3 ± 0.32 kg, were used in this study. Experimental OA in rabbits was induced by anterior cruciate ligament transection (ACLT) surgery [15]. All animals were kept in an animal experimental unit at a temperature of 22 to 25°C, a humidity of 50% to 60%, and with a 12-h day/night cycle. The animals had free access to their respective diets and water; feed consumption was checked twice a week. The health of the animals was checked daily, and body weights were monitored twice a week. The experimental protocol was approved by the Faculty of Veterinary Medicine and the Ethics Committee, Chiang Mai University, Thailand.

Animals were randomly separated into four groups. Group 1 (G.1) was not treated, group 2 (G.2) was treated with 0.5% dry pineapple shell, group 3 (G.3) was treated with 1.0% dry pineapple shell, and group 4 (G.4) was treated with 100 mg glucosamine sulphate (Synoquin®, T.J. Animal Health, Bangkok, Thailand).

2.2 Anterior Cruciate Ligament Transection

All rabbits were anesthetized by intravenous injection of 10 mg/kg tiletamine+zolazepam (Zoletil®, Virbac Laboratories, Carros, France). Each animal was subjected to transection of the ACL of the right knee, according to previous reports [15]. Briefly, after shaving and sterilizing the surgical site, ACLT was performed using a paramedial approach with the skin incision in the left knee

medial parapatellar area. To achieve optimal visualization of the anterior cruciate ligament, the patellar bone was displaced laterally and the knee was placed in full flexion. The anterior stability was confirmed by an anterior drawer test. The synovium and the incised skin were sutured and sterile dressing was applied. Following the surgical procedure, 20 mg/kg cefazolin (Nida Pharma Inc., Bangkok, Thailand) was injected intramuscularly into each rabbit twice daily for 1 week.

2.3 Biomarker Assay

One ml blood samples were collected from the ear vein, once a week for two weeks before the operation, and every two weeks after the operation for 12 weeks. These blood samples were used for serum biomarker measurement by enzyme-linked immunoassays (ELISA), as previously described [2,16,17]. This study used two biomarkers: chondroitin sulfate WF6 epitope (CS-WF6) and hyaluronan (HA).

2.3.1 Competitive immunoassay using monoclonal antibody WF6

A mouse monoclonal antibody WF6 was raised against a shark cartilage aggrecan preparation; a quantitative ELISA for the epitope, recognized by monoclonal antibody WF6, was modified from previous reports [2,16,17]. The antibody was specific for intact chondroitin sulfate chains and showed no interaction with other sulfated glycosaminoglycans, hyaluronan, or other polyanions such as DNA, RNA or dextran sulfate. The standard used in the assay was shark cartilage aggrecan (A1 fraction) at concentrations of 19-10,000 ng/ml in 6% bovine serum albumin (BSA) in TE buffer (0.1 M Tris HCl, pH 7.4, containing 0.15 M sodium chloride, 0.1% Tween 20 and 0.1% BSA). Diluted human serum samples (1:5 in 6% BSA-TE) were added to 1.5 ml plastic tubes containing an equal volume of WF6 (cell culture supernatant, 1:200 dilution in TE buffer). They were incubated at 37°C for 1 h, and then placed on a microtiter plate, which was pre-coated with shark aggrecan (A1 fraction). Non-specific protein binding was blocked with BSA. The plates were incubated at 37°C for 1 h; the wells were then washed, and peroxidase-conjugated anti-mouse IgM antibody (1:2,000) was added (100 ml/well, in TE buffer). The bound conjugate was detected by adding ortho-phenylenediamine (*o*-PD) substrate (100 ml/well, in 0.05 M citrate buffer, pH 5.0). The reaction was stopped after 10 min with 50 ml/well of 4 M sulfuric acid. Absorbance was determined using a microplate reader at 492/690 nm. The concentration of WF6 epitope in supernatant samples was calculated by reference to a standard curve.

2.3.2 ELISA-based assay for HA using biotinylated HA-binding proteins (HABPs)

Human serum samples or standard HA (HealonR) at various concentrations (19-10,000 ng/ml) in 6% bovine serum albumin-phosphate buffered saline (BSA-PBS), pH 7.4, were added to 1.5 ml plastic tubes containing biotinylated HA-binding proteins (HABPs), prepared as described above (1:200 in 0.05 M Tris-HCl buffer, pH 8.6). The tubes were incubated at room temperature for 1 h; samples were then added to the microplate, which was pre-coated with umbilical cord HA (100 ml/well of 10 mg/ml) and blocked with 1% BSA (150 ml/well). The plate was then incubated at room temperature for 1 h. The wells were then washed, and peroxidase-conjugated anti-biotin antibody (1:2,000 dilution), 100 ml/well in PBS, was added. The plate was incubated at room temperature

for another 1 h. The detection of conjugated antibody was with *o*-PD substrate; plate reading was carried out as described above. The concentration of HA in samples was calculated from the standard curve [2,16,17].

2.4 Animal Euthanasia and Tissue Preparation

At the 12th week of the experiment, 3 animals from each group (12 animals) were randomized for euthanasia using an overdose of pentobarbital (Ceva Animal Health, Libourne, France). Both stifle joints (right, experimental; left, control) were explored for gross evaluation, and cartilage collected for histological evaluation.

After the articular cartilage had been scored, the femoral condyles of the rabbits were fixed in 10% neutral buffered formalin. Each sample was placed into 15% disodium EDTA decalcifying solution (pH 7.4) and shaken at 4°C. Decalcifying solution was changed three times each week for 4 weeks. The specimens were rinsed thoroughly, dehydrated, and embedded in paraffin at 60°C. Seven-μm-thick sections were prepared and stained with hematoxylin and eosin (H&E), and Safranin O for microscopic examination of the tissue [18].

2.5 Histopathological Examinations

The quality of the articular cartilage tissue was evaluated by two methods: gross anatomy and histology. Prior to tissue collection, articular cartilage was double-blindly scored by veterinary orthopedic surgeons using the system proposed by Duc and coworkers [19], as shown in Table 1. Histopathological examinations were double-blindly scored by pathologists according to the scoring system proposed by Oegema and coworkers [20], as shown in Table 2.

Table 1. Gross anatomy score of articular cartilage [19].

Score	Criteria
0	normal cartilage
1	cartilage softening without surface abnormalities
2	erosions or fissures with a depth of not more than 50% of the cartilage thickness
3	erosions or fissures with a depth of greater than 50% but less than 100%;
4	cartilage lesion to the subchondral bone

Table 2. Histological analysis of cartilage [20].

Score	Criteria
0	Articular cartilage smooth, uninterrupted
1	Minimal/mild superficial fibrillation (≤1/10 of articular cartilage thickness) involving <half of the plateau, condyle or groove
2	Minimal/mild superficial fibrillation (≤1/10 of articular cartilage thickness) involving ≥half of the plateau, condyle or groove
3	Fibrillation/clefts/loss of articular cartilage involving superficial one-third of articular cartilage in <half of the plateau, condyle or groove
4	Fibrillation/clefts/loss of articular cartilage involving superficial one-third of articular cartilage in ≥half of the plateau, condyle or groove
5	Fibrillation/clefts/loss of articular cartilage involving superficial one-third to two-thirds of articular cartilage in <half of the plateau, condyle or groove

Table 2. continued

Score	Criteria
6	Fibrillation/clefts/loss of articular cartilage involving superficial one-third to two-thirds of articular cartilage in ≥half of the plateau, condyle or groove
7	Fibrillation/clefts/loss of articular cartilage involving >two-third depth of articular cartilage in <half of the plateau, condyle or groove
8	Fibrillation/clefts/loss of articular cartilage involving >two-third depth of articular cartilage in ≥half of the plateau, condyle or groove
9	Fibrillation/clefts/loss of articular cartilage to subchondral bone involving <half of the plateau, condyle or groove
10	Fibrillation/clefts/loss of articular cartilage to subchondral bone involving ≥half of the plateau, condyle or groove

2.6 RNA Isolation, Synthesis of cDNA

RNA isolation and purification in each group was performed using an RNeasy Mini Kit protocol (Qiagen, Hilden, Germany), including the DNA removal step, according to the manufacturer's guidelines. RNA was eluted in 40 µl of RNase-free water (Qiagen). Reverse transcription was performed using 10 µl RNA with oligo(dT)12-18 and SuperScript II reverse transcriptase (Invitrogen, Karlsruhe, Germany). First, mRNA and oligo(dT) primer were mixed, heated to 70°C for 3 min, and placed on ice until the addition of the remaining reaction components. The reaction was incubated at 42°C for 90 min, and terminated by heat inactivation at 70°C for 15 min.

2.7 Quantitative Real-time PCR

Quantification of six transcripts - type II collagen (Col-2), interleukin-1 beta (IL-1β), matrix metallopeptidase-1 and -3 (MMP-1, MMP-3), tissue inhibitor of metallopeptidase-1 (TIMP-1), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Shanghai BlueGene Biotech Co. Ltd., Shanghai, China) - was performed in all four groups. An ABI Prism® 7000 apparatus (Applied Biosystems, Foster City CA, USA) was used for quantitative analysis using SYBR® Green JumpStart™ Taq ReadyMix™ (Sigma-Aldrich, St. Louis MO, USA), incorporating dsDNA-specific fluorescent detection dye. Quantitative analyses of cell Col-2, IL-1β, MMP-1, MMP-3, and TIMP-1 cDNA were performed in comparison with GAPDH as an endogenous control, and were run in separate wells. PCR was performed by using 2 µl of each sample of cDNA and specific amplification primers. The primer sequences were designed for PCR amplification according to the human cDNA sequence (Table 3) using Primer Express® software v2.0 (Applied Biosystems). Standard curves were generated for both target and endogenous control genes using serial dilution of plasmid DNA (10^1-10^8 molecules). The PCRs were performed in 20 µl reaction volume containing 10.2 µl SYBR® Green universal master mix, optimal levels of forward and reverse primers, and 2µl of embryonic cDNA. During each PCR, reaction samples from the same cDNA source were run in duplicate to control the reproducibility of the results. A universal thermal cycling parameter (an initial denaturation step at 95°C for 10 min, and 45 cycles of denaturation at 95°C for 15s and 60°C for 60 s) was used to quantify each gene of interest. After the end of the last cycle, a dissociation curve was generated by starting the fluorescence acquisition at 60°C and

taking measurements at 7 s intervals until the temperature reached 95°C. Final quantitative analysis was done using the relative standard curve method, as in previous reports [11,12]. Results were reported as the relative expression level compared to the calibrator cDNA after normalization of the transcript amount to the endogenous control.

Table 3. Set of primers used for real-time quantitative PCR.

Gene	Accession number	Sequences	Base pairs
Col-2	NM_001195671	5'-GCTCAAGTCCCTCAACAACCAG-3'	131
		5'-GGTCTATCCAGTAGTCACCGCTCT-3'	
IL-1β	M26295	5'-TGCTGTCCAGACGAGGGCAT-3'	293
		5'-ACTCTCCAGCTGCAGGGTAG-3'	
TIMP-1	J04712	5'-ACCTTGTCATCAGGGCCA-3'	350
		5'-ACAGGCAAACACTGTGCA-3'	
MMP-1	M17821	5'-TCAGTTCGTCCTCACTCCAG-3'	649
		5'-TTGGTCCACCTGTCATCTTC-3'	
MMP-3	M25664	5'-AAGTTCCTTGGCTTGGAGGT-3'	308
		5'-ATCTCCATGTTCTCGGACTC-3'	
GAPDH	L23961	5'-TCACCATCTTCCAGGAGCGA-3'	293
		5'-CACAATGCCGAAGTGGTCGT-3'	

2.8 Western Blot Analysis

Western blot analysis was used to study the expression of Col-2, IL-1β, MMP-1, MMP-3 and TIMP-1 protein in the right and left articular cartilage in all experimental groups. For this, 50 µl protein samples were extracted from the cartilage in loading buffer: 26% of Tris 1 M (pH 6.8), 12% of SDS, 20% of 2-mercaptoethanol, and 40% of glycerol. After boiling for 5 min, proteins were separated on a 10% SDS-PAGE gel. Proteins were then transferred onto a Protran® nitrocellulose transfer membrane (Schleicher & Schuell Bio Science, Dassel, Germany), pore size 0.45 µm, using a Trans-Blot® SD semi-dry transfer cell (Bio-Rad Laboratories, Hercules CA, USA). The membrane was stained with Ponceau S to evaluate the transfer quality, and blocked for 1 h in Tris-buffered saline (20 mM Tris, pH 7.5; 150 mM NaCl) containing 0.05% Tween-20 (TBS-T) and 1% polyvinylpyrrolidone (PVP) (Sigma-Aldrich). The membrane was then incubated at 4°C overnight with primary antibody – rabbit anti-collagen type II (1:250), MMP-1 (1:150), MMP-3 (1:200), and TIMP-1 (1:200) - which were diluted in TBS-T containing 0.1% PVP prior to use. After incubation with the primary antibody, the membrane was washed six times (10 min each) in TBS-T. Hybridization with the secondary antibody was performed at room temperature for 1 h. The secondary antibody, horseradish-peroxidase-conjugated donkey anti-rabbit or anti-mouse secondary antibody (Amersham Biosciences, Buckinghamshire, UK), was diluted 1:50,000 in TBS-T containing 0.1% PVP. The membrane was finally washed six times (10 min each) in TBS-T. Peroxidase activity was detected using the ECL Plus Western Blotting Detection System (Amersham Biosciences), following the manufacturer's instructions, and visualized using Kodak BioMax XAR film (Kodak,

Rochester NY, USA). Immunoblots were further semi-quantitatively analyzed using the computer program Quantity One® (Bio-Rad Laboratories). The mean optical density represented the relative expression levels of protein. Experiments were performed in triplicate.

2.9 Statistical Analysis

General identification information and cartilage score assessments of the four groups, as well as semi-quantitative Western blot experiments and quantitative data of serum WF6 and HA were analyzed by a non-parametric Wilcoxon rank-sum test. The mRNA expression analysis for studied genes in all treatment groups was based on the relative standard curve method. All statistical analysis was performed with the STATA software program, version 10.0; $P<0.05$ was considered statistically significant.

3. RESULTS

3.1 Composition of Pineapple Shell

The pineapple shell was composed of 17.58 ± 1.02% dry matter (DM), 5.46 ± 1.08% crude protein (CP), 2.67 ± 0.09% ether extract (EE), 14.16 ± 0.86% crude fiber (CF), 4.89 ± 0.32% ash, 72.81 ± 2.3% nitrogen-free extract (NFE), 25.48 ± 2.18% hemicellulose, 18.14 ± 0.89% cellulose, and 1.82 ± 0.08 lignin.

3.2 Level of Serum Biomarker

Both serum biomarkers were significantly changed ($P<0.05$) after the operation. The level of CS-WF6 was increased while HA was decreased.

The levels of CS-WF6 in the treated groups (G.2 and G.3) were not different ($P>0.05$) when compared to the control group (G.1). The levels of CS-WF6 were significantly increased ($P<0.05$) from week 2, and persisted throughout the remainder of the experiment. At the end of the experiment (week 12), serum CS-WF6 level was 5-fold higher than the starting (basal) level (Table 4).

In parallel with CS-WF6 level, the levels of HA in G.2 and G.3 were not different ($P>0.05$) from that of G.1. HA levels were significantly decreased ($P<0.05$) from week 2, and persisted throughout the remainder of the experiment. At the end of the experiment (week 12), serum HA level was lower than the baseline level by approximately 0.5- 0.6-fold. In G.4, the level of HA increased significantly throughout the experiment, with an increase in the final level of 0.7-fold (Table 5).

Table 4. Relative change of serum chondroitin sulfate epitope WF6.

Weeks	Groups			
	1 (control)	2 (0.5%)	3 (1.0%)	4 (100mg GS)
0	99.50 ± 10.81^{a}	104.00 ± 12.65^{a}	108.50 ± 14.79^{a}	104.25 ± 10.57^{a}
2	135.00 ± 11.75^{b}	137.38 ± 15.39^{b}	133.13 ± 16.46^{b}	129.25 ± 9.27^{b}
4	318.63 ± 21.66^{c}	327.25 ± 27.60^{c}	301.63 ± 24.37^{c}	164.38 ± 19.29^{c*}
6	279.13 ± 38.01^{d}	309.00 ± 21.74^{c}	274.38 ± 32.20^{c}	186.25 ± 28.47^{c*}
8	438.13 ± 33.52^{e}	430.25 ± 33.26^{d}	401.13 ± 16.53^{d}	155.25 ± 16.41^{c*}
10	489.38 ± 28.64^{f}	503.50 ± 22.28^{d}	464.38 ± 25.73^{e}	156.38 ± 9.71^{c*}
12	536.88 ± 29.13^{g}	545.75 ± 24.71^{d}	504.63 ± 18.42^{f}	152.00 ± 15.91^{c*}

Different letters (a-g) mean significant difference between weeks at the same group ($P<0.05$), symbol (*) mean significant difference between groups at the same weeks ($P<0.05$)

Table 5. Relative change of serum hyaluronan.

Weeks	Groups			
	1 (control)	2 (0.5%)	3 (1.0%)	4 (100mg GS)
0	100.38 ± 4.87[a]	102.75 ± 3.38[a]	98.25 ± 10.98[a]	98.88 ± 6.33[a]
2	88.00 ± 5.73[b]	86.50 ± 8.05[b]	84.63 ± 7.65[b]	95.50 ± 9.20[a]
4	69.50 ± 7.27[c]	66.00 ± 8.05[c]	73.25 ± 4.95[c]	115.00 ± 9.93[a,b]
6	45.00 ± 10.68[d]	46.63 ± 6.35[d]	57.50 ± 9.99[d]	128.38 ± 14.04[b]
8	43.00 ± 6.87[d]	53.13 ± 7.88[e]	51.75 ± 7.00[d]	128.25 ± 7.27[b]
10	44.50 ± 7.96[d]	51.75 ± 8.75[e]	62.50 ± 5.66[d]	150.75 ± 12.79[c]
12	39.63 ± 6.84[d]	50.75 ± 10.73[e]	55.88 ± 6.33[d]	172.88 ± 18.17

Different letters (a-g) mean significant difference between weeks at the same group (P<0.05), symbol (*) mean significant difference between groups at the same weeks (P<0.05)

3.3 Histopathological Score

The results of histopathological studies on the control and experimental groups are shown in Table 6 and Figure 1. Gross anatomical scores of the right stifle joint were not different among G.1, G.2 and G.3 (3.25, 3.33 and 3.17, respectively), whereas a significantly different score was found in G.4 (1.92). The histological scores were in agreement with the gross anatomy scores (G.1 = 8.75, G.2 = 8.42, G.3 = 8.00, G.4 = 5.83).

Table 6. Anatomy and histology score.

	Stifle joint	Groups			
		1 (control)	2 (0.5%)	3 (1.0%)	4 (100mg GS)
Anatomy	Right	3.25 ± 0.62	3.33 ± 0.49	3.17 ± 0.58	1.92 ± 0.67*
	Left	0.00 ± 0.00¶	0.00 ± 0.00¶	0.00 ± 0.00¶	0.00 ± 0.00¶
Histology	Right	8.75 ± 0.75	8.42 ± 1.08	8.00 ± 1.28	5.83 ± 1.47*
	Left	0.08 ± 0.29¶	0.08 ± 0.32¶	0.00 ± 0.00¶	0.00 ± 0.00¶

* mean significant difference between groups at the same joint (P<0.05)

¶ mean significant difference between joint (right and left) at the same group (P<0.05)

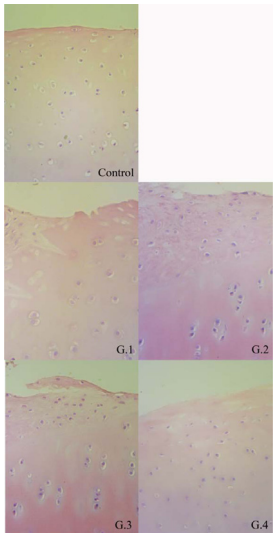

Figure 1. Low-power field microscopic evaluation of articular cartilage (H&E, x10) representative samples from each group (G.1-4).

3.4 Gene Expression Profiles

Firstly, all gene expressions of articular cartilage showed a significant difference ($P<0.05$) when comparing the surgical (right) and non-surgical (left) stifle joints (Figure 2). Relative expressions of IL-1β, MMP-3 and MMP-1 in surgical joints were 5-, 3- and 4-fold higher than in non-surgical joints, respectively; while the relative expressions of Col-2 and TIMP-1 in surgical joints were 0.75-fold lower, respectively, than in non-surgical joints.

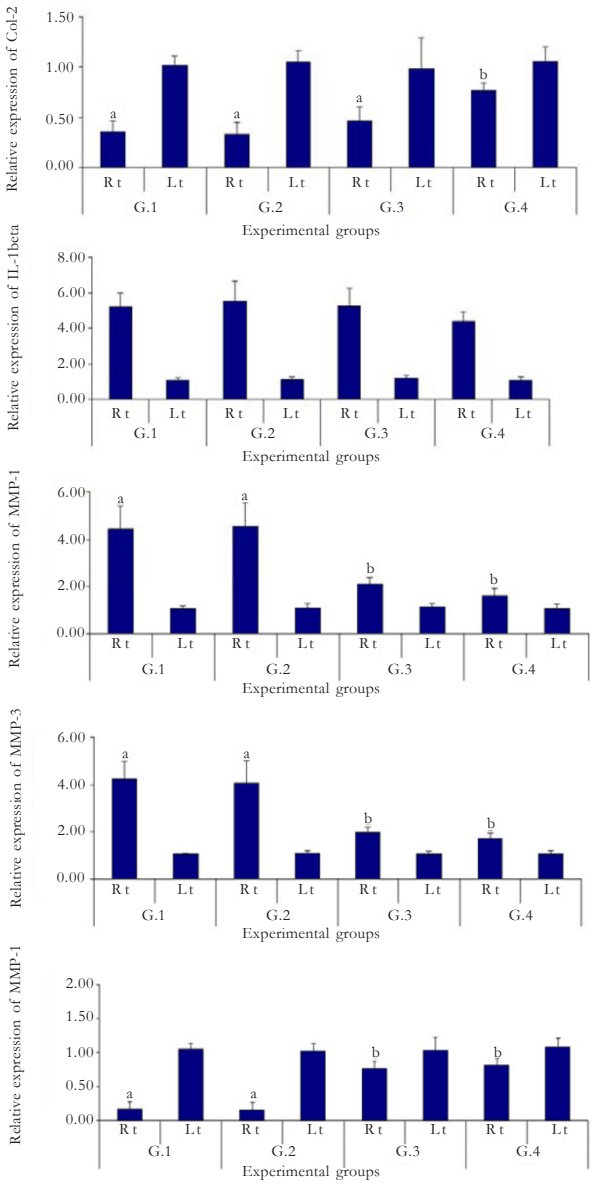

Figure 2. Relative expression of mRNAs assessed by real-time PCR. The mRNA expression levels of IL-1beta, MMP-3 and MMP-1 were significantly increased in right knee of all groups. The mRNA expression levels of Coi-2 and TIMP-3 were significantly decreased in right knee of all groups ($P < 0.05$). A significant difference ($P < 0.05$) between the four groups at the same joint (right and left) is displayed with superscript (^{a,b}) on the bars.

IL-1β gene expression did not differ significantly among surgical joints in the four experimental groups. The expressions of MMP-1 and MMP-3 in G.3 and G.4 were higher than in G.1 and G.2 ($P<0.05$). In contrast, the expression of Col-2 in G.1, G.2 and G.3 were lower than in G.4 ($P<0.05$). The expressions of TIMP-1 in G.3 and G.4 were lower than in G.1 and G.2 ($P<0.05$).

3.5 Protein Expression Using Western Blot

The semi-quantitative analysis of protein expression is shown in Table 7. The expressions of collagen type 2 and TIMP-1 protein in G.4 were significantly higher than in the other three groups (G.1-G.3). A comparison among the experimental groups found that expression in the non-operated joint (left knee) in G.1-G.3 was significantly higher than in the operated joint (right knee); whereas in G.4 there was no significant difference between the two joints ($P>0.05$).

The expressions of IL-1β, MMP-1 and MMP-3 had the same pattern. These proteins in all three groups (G.1-G.3) were significantly increased in the operated joint. The expressions of IL-1β and MMP-1 protein showed a significant difference between the operated and non-operated joints in all four groups. In G.1-G.3, MMP-3 protein in the operated joint was significantly higher ($P<0.05$) than in the non-operated joint.

Table 7. Immunoblot analysis for the expression of Col-2, IL-1beta, MMP-1, MMP-3 and TIMP-1 protein.

Protein	Stifle joint	Groups			
		1 (control)	2 (0.5%)	3 (1.0%)	4 (100mg GS)
Col-2	Right	0.29 ± 0.07	0.37 ± 0.15	0.30 ± 0.11	0.79 ± 0.08*
	Left	1.03 ± 0.08¶	1.06 ± 0.16¶	1.01 ± 0.11¶	1.05 ± 0.08
IL-1beta	Right	1.04 ± 0.18	0.97 ± 0.08	0.92 ± 0.13	0.25 ± 0.09*
	Left	0.12 ± 0.03¶	0.10 ± 0.02¶	0.09 ± 0.03¶	0.09 ± 0.02¶
MMP-1	Right	0.96 ± 0.08	0.88 ± 0.12	0.94 ± 0.11	0.20 ± 0.05*
	Left	0.16 ± 0.07¶	0.15 ± 0.06¶	0.11 ± 0.04¶	0.10 ± 0.04¶
MMP-3	Right	1.03 ± 0.09	0.97 ± 0.11	0.95 ± 0.15	0.32 ± 0.08*
	Left	0.16 ± 0.04¶	0.27 ± 0.04¶	0.23 ± 0.09¶	0.22 ± 0.05
TIMP-1	Right	0.33 ± 0.07	0.34 ± 0.09	0.52 ± 0.08	0.95 ± 0.07*
	Left	0.92 ± 0.11¶	0.93 ± 0.06¶	0.97 ± 0.07¶	1.02 ± 0.09

* mean significant difference between groups at the same joint ($P<0.05$)

¶ mean significant difference between joint (right and left) at the same group ($P<0.05$)

4. DISCUSSION

Currently, a cure for OA remains difficult to achieve. OA management is mainly concerned with the alleviation of symptoms. OA management recommendations include a combination of pharmacological treatment and non-pharmacological management. Current pharmacological treatments, such as alternative nutrition-based treatment, have become popular. A chronic disease such as OA is the most suitable pathological model for nutrition treatment. Natural nutritional treatments are more appropriate for providing long-term health benefits than other short-term treatments. Hence, nutraceuticals/functional food could be a promising alternative.

Bromelain is a complex natural

mixture of proteolytic enzymes derived from pineapple stem. Its functions include inhibition of malignant cell growth, anti-thrombus formation, suppression of inflammation, control of diarrhea, promotion of skin debridement, and so forth [22,23]. In some countries, bromelain can be purchased as a health/nutritional supplement. Due to its anti-inflammatory and analgesic properties, bromelain may serve as an alternative treatment to NSAIDs for patients with osteoarthritis [23]. Mostly the stems and pulp of pineapples are used for bromelain extraction. However, pineapple peel is reported to be a potential source of bromelain because of the large amount of waste after processing [24]. Previous study reported that the quantity of bromelain from peel was smaller than from the stem; and the enzyme activity of bromelain from the shell was the lowest compared to the core, crown and stem of pineapple [23,24]. Administration of 0.5% (600 mg) and 1.0% (1,200 mg) of dry pineapple shell for 3 months might not provide a sufficient amount of bromelain to improve biomarker levels and histological scores of the joints in OA rabbits; while the glucosamine ulphate (GS)-treated group showed significant improvement of gross and histological scores.

In the present study, three gene expression profiles (MMP-1, MMP-3 and TIMP-1) from G.3 were significantly different from G.1; but the levels of these proteins were not different. The observed level of protein expression was not related to gene expression level, suggesting the importance of post-transcriptional mechanisms in controlling the initiation and translation of protein synthesis [25]. In normal cartilage, all active MMPs are inhibited by tissue inhibitors of metalloproteinase (TIMPs), which bind tightly to active MMPs in a 1:1 ratio [26]. The degradation of cartilage substrates in OA could be characterized as an imbalance between the amount of TIMPs and MMPs [21]. The ratio between MMP-3 and TIMP-1 in G.3 was more than 1, which revealed that gene expression of MMP-3 was higher than TIMP-1. As a result of the high level of MMP-3 protein, the breakage of cartilage substrates was shown via high anatomical and histological scores (Table 6).

In the glucosamine sulfate (GS) group, the ratio of MMP-3 and TIMP-1 was higher than 1; but the level of TIMP-1 protein was higher than MMP-3. When TIMP levels exceed those of active enzymes (MMPs), connective tissue turnover is prevented [27], resulting in the lowest scores for anatomy and histology, as opposed to G.1-G.3. GS - one form of glucosamine available for clinical trials - has been used extensively for pain management in OA. Glucosamine also promotes the regeneration of cartilage, including modulating the level of the extracellular matrix component, and increases the inhibiting substance of proteolytic enzymes (TIMP) [27,28]. Interestingly, glucosamine is safe, with little or no side-effects when appropriately used [29]. For these reasons, using glucosamine as a positive control in this study helped to confirm the potential use of glucosamine in OA therapy.

5. CONCLUSIONS

This was a preliminary study of pineapple shell as a supplementary food for treatment of OA in rabbits. Neither level of dry pineapple shell (0.5 and 1.0%) altered the degradation of articular cartilage in rabbits with experimentally induced OA. However, the gene expression pattern of MMP-1 and MMP-3 in G.3 (1.0% dry pineapple shell) was significantly decreased, while an increased level of TIMP-1 expression was observed. Supplementation with higher dosages and for longer periods of study should be considered before future application in clinical trials.

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REFERENCES

[1] Johnston S.A., Osteoarthritis: joint anatomy, physiology and pathobiology, *Vet. Clin. North Am. Small Anim. Pract.*, 1997; **27**: 699-723.

[2] Nganvongpanit K., Pothacharoen P., Suwankong N., Ong-Chai S. and Kongtawelert P., The effect of doxycycline on canine hip osteoarthritis: design of a 6-months clinical trial, *J. Vet. Sci.,* 2009; **10**: 239-247.

[3] Monfort J., Pelletier J.P., Garcia-Giralt N. and Martel-Pelletier J., Biochemical basis of the effect of chondroitin sulphate on osteoarthritis articular tissues, *Ann. Rheum. Dis.,* 2008; **67**: 735-740.

[4] Nganvongpanit K., Pothacharoen P., Chaochird P., Klunklin K., Warrit K., Settakorn J., Pattamapaspong N., Luevitoonvechkij S., Arpornchayanon O., Kongtawelert P. and Pruksakorn D., Prospective evaluation of serum biomarker levels and cartilage repair by autologous chondrocyte transplantation and subchondral drilling in a canine model, *Arthritis Res. Ther.*, 2009; **11**: R78.

[5] Halket A., Stratford P.W., Kennedy D.M. and Woodhouse L.J., Using hierarchical linear modeling to explore predictors of pain after total hip and knee arthroplasty as a consequence of osteoarthritis, *J. Arthroplasty,* 2010; **25**: 254-262.

[6] German B., Schiffrin E.J., Reniero R., Mollet B., Pfeifer A. and Neeser J.R., The development of functional foods: lessons from the gut, *Trends. Biotechnol.,* 1999; **17**: 492-499.

[7] Maurer H.R., Bromelain: biochemistry, pharmacology and medical use, *Cell. Mol. Life Sci.,* 2001; **58**: 1234-1245.

[8] Cohen A. and Goldman J., Bromelains therapy in rheumatoid arthritis**,** *Pa. Med. J.,* 1964; **67**: 27-30.

[9] Walker A.F., Bundy R., Hicks S.M. and Middleton R.W., Bromelain reduces mild acute knee pain and improves well-being in a dose-dependent fashion in an open study of otherwise healthy adults, *Phytomedicine*, 2002; **9**: 681-686.

[10] Leipner J., Iten F. and Saller R., Therapy with proteolytic enzymes in rheumatic disorders, *Biol. Drugs.*, 2001; **15**: 779-789.

[11] Siengdee P., Nganvongpanit K., Pothacharoen P., Chomdej S., Mekchay S. and Ong-Chai S., Effects of bromelain on cellular characteristics and expression of selected genes in canine *in vitro* chondrocyte culture, *Vet. Med-Czech.,* 2010; **55**: 551-560.

[12] Gagnier J.J., Chrubasik S. and Manheimer E., Harpgophytum procumbens for osteoarthritis and low back pain: A systematic review, *BMC Complement Altern. Med.,* 2004; **4**: 13.

[13] Deodhar S.D., Sethi R. and Srimal R.C., Preliminary study on antirheumatic activity of curcumin (diferuloyl methane), *Indian J. Med. Res.,* 1980; **71**: 632-634.

[14] Altman R.D. and Marcussen K.C., Effects of a ginger extract on knee pain in patients with osteoarthritis, *Arthritis Rheum.*, 2001; **44**: 2531-2538.

[15] Jean Y.H., Wen Z.H., Chang Y.C.,

Hsieh S.P., Lin J.D., Tang C.C., Chen W.F., Chou A.K. and Wong C.S., Increase in excitatory amino acid concentration and transporters expression in osteoarthritic knees of anterior cruciate ligament transected rabbits, *Osteoarthritis Cartilage,* 2008; **16**: 1442-1449.

[16] Pothacharoen P., Teekachunhatean S., Louthrenoo W., Yingsung W., Ong-Chai S., Hardingham T. and Kongtawelert P., Raised chondroitin sulfate epitopes and hyaluronan in serum from rheumatoid arthritis and osteoarthritis patients, *Osteoarthritis Cartilage,* 2006; **14**: 299-301.

[17] Nganvongpanit K., Itthiarbha A., Ong-Chai S. and Kongtawelert P., Evaluation of serum chondroitin sulfate and hyaluronan: Biomarkers for osteoarthritis in canine hip dysplasia, *J. Vet. Sci.,* 2008; **9**: 317-325.

[18] Bentley G., Biant L.C., Carrington R.W.J., Akmal M., Goldberg A., Williams A.M., Skinner J.A. and Pringle J., A prospective, randomised comparison of autologous chondrocyte implantation versus mosaicplasty for osteochondral defects in the knee, *J. Bone Joint Surg.,* 2003; **85**: 223-230.

[19] Duc S.R., Koch P., Schmid M.R., Horger W., Hodler J. and Pfirrmann C.W.A., Diagnosis of articular cartilage abnormalities of the knee: Prospective clinical evaluation of a 3D water-excitation true FISP sequence, *Radiology,* 2007; **243**: 475-482.

[20] Oegema T.R., Carlson C.S. and Cole A.A., Histological analysis of cartilage conditions, in An Y.H., Martin K.L. and Totowa N.J., eds., Handbook of Histology Methods for Bone and Cartilage, Humana Press, 2003: 423-436.

[21] Nganvongpanit K., Chaochird P., Siengdee P., Pothacharoen P., Klunklin K., Chomdej S., Mekchay S. and Kongtaweelert P., *In vitro* suppression of the MMP-3 gene in normal and cytokine-treated human chondrosarcoma using small interfering RNA. *J. Orthop. Surg. Res.,* 2009; **4:** 45.

[22] Tochi B.N., Wang Z., Xu S.Y. and Zhang W., Therapeutic application of pineapple protease (bromelain): A review, *Pakistan. J. Nutr.,* 2008; **7:** 513-520.

[23] Sriwatanapongse A., Balaban M. and Teixeira A., Thermal inactivation kinetics of bromelain in pineapple juice, *Trans. ASAE.,* 2000; **43**: 1703-1708.

[24] Hebbar H.U., Sumana B. and Raghavarao K.S.M.S., Use of reverse micellar systems for the extraction and purification of bromelain from pineapple wastes, *Bioresour. Technol.,* 2008; **99**: 4896-4902.

[25] Cawston T.E. and Wilson A.J., Understanding the role of tissue degrading enzymes and their inhibitors in development and disease, *Best. Pract. Res. Clin. Rheumatol.,* 2006, **20**: 983-1002.

[26] Blanco Garcia F.J., Catabolic events in osteoarthritic cartilage, *Osteoarthritis Cartilage,* 1999; **7**: 308-309.

[27] Dodge G.R. and Jimenez S.A., Glucosamine sulfate modulates the levels of aggrecan and matrix metalloproteinase-3 synthesized by cultured human osteoarthritis articular chondrocytes, *Osteoarthritis Cartilage,* 2003; **11:** 424-432.

[28] Uitterlinden E.J., Koevoet J.L.M., Verkoelen C.F., Bierma-Zeinstra S.M.A., Jahr H., Weinans H., Verhaar J.A.N. and van Osch G.J.VM., Glucosamine increases hyaluronic acid production in human osteoarthritic synovium explants, *BMC Musculoskelet Disord.*, 2008; **11:** 120.

[29] Anderson J.W., Nicolosi R.J. and Borzelleca J.F., Glucosamine effects in humans: A review of effects on glucose metabolism, side effects, safety considerations and efficacy, *Food Chem. Toxicol.,* 2005; **43:** 187-201.