Effects of bromelain on cellular characteristics and expression of selected genes in canine *in vitro* chondrocyte culture

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ABSTRACT: The purpose of this study was to determine the effect of bromelain treatment on canine articular chondrocytes *in vitro*. This research evaluated cell viability, levels of apoptotis and mitotis, proteoglycan concentrations and the expression of certain genes. Chondrocytes were exposed to 50 µg/ml bromelain for 4, 16 and 32 h. The rate of apoptotis in the treatment groups was significantly lower than in the control groups that were incubated with media only ($P < 0.05$); and the mitotic rate in treatment groups was significantly higher than in the control groups ($P < 0.05$), at all durations of exposure. The effect of bromelain on gene expression was measured by the real-time PCR technique. It was found that bromelain significantly decreased ($P < 0.05$) TIMP-1 and MMP-3 expression. These experimental bromelain treatments have shown positive results, and have increased the basic knowledge in regard to the healing and modulation of osteoarthritis, prior to the general use of bromelain in clinical practice.

Keywords: bromelain; chondrocyte; dog; cysteine proteinase

Osteoarthritis (OA) is the one of most important orthopaedic diseases. It is characterized by progressive degradation of articular cartilage and the loss of chondrocytes, which has been proposed as the critical signal for initiation and progression of OA. Moreover, OA results in impaired joint motion and pain. Its high prevalence and its moderate-to-severe impact on daily life pose a significant public health problem in humans as well as pets (Goldring, 2000; Nganvongpanit and Ong-Chai, 2004). Currently, the management of OA includes a combination of nonpharmacological and pharmacological treatments, such as non-steroidal anti-inflammatory drugs (NSAIDs). Chondroprotective drugs are currently undergoing clinical trials for OA, but their side effects have not been determined (Johnston and Budsberg, 1997). In recent years, plant extracts and phytotherapeutic drugs have been used as alternative treatments. They may play a beneficial role in some diseases, and have few undesirable side effects (German et al., 1999). A wide range of therapeutic benefits has been claimed for bromelain, which is extracted from pineapple stems (*Ananas comosus*) and is a member of the cysteine proteinase group. Bromelain also contains a peroxidase, acid phosphatase, several protease inhibitors, and organically bound calcium (Kelly, 1996; Maurer, 2001). Bromelain activity is stable over a wide pH

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range, and has shown potentially beneficial effects due to its anti-inflammatory and analgesic properties. Its several therapeutic properties – including anti-tumour action, modulation of cytokines and immunity, skin debridement, and enhanced wound healing – have been demonstrated in both *in vitro* and *in vivo* studies (Cohen and Goldman, 1964; Maurer, 2001; Shahid et al., 2002). Currently, bromelain is a food supplement that may provide an alternative treatment to NSAIDs for patients with OA. Many studies have shown good potential for bromelain in the treatment of OA, however, due to methodological issues these have not produced definitive conclusions (Maurer, 2001; Walker et al., 2002; Brien et al., 2004; Tochi et al., 2008). To date there is no published report concerning the efficacy of bromelain on chondrocyte cells, which would be a necessary basis for the informed use of bromelain in medical practice.

Therefore, the purpose of the present study was to determine the effects of bromelain treatment on canine chondrocyte culture *in vitro* by investigating cell morphology, mRNA expression and glycosaminoglycan (GAG) productivity. The potential therapeutic benefits of using bromelain instead of anti-inflammatory and chondroprotective drugs should be followed up in further studies.

MATERIAL AND METHODS

Reagents. Bromelain lyophilized powder was obtained from Sigma (St. Louis, USA) No. B5144. Bromelain was diluted in a medium containing DMEM (Gibco, USA) pH 7.4 without serum and stored at –20 °C until treatment.

Isolation of cells and cell culture. Canine articular cartilage samples were collected from the femoral condyle of non-OA stifle joints (from dogs aged three to five years old and weighing about 10–20 kg) at the Small Animal Hospital, Faculty of Veterinary Medicine, Chiang Mai University, Thailand. The patients had suffered a car accident or disease at the time of limb amputation (for example, osteosarcoma). To isolate chondrocytes by enzymatic treatment according to the method of Frohlich et al. (2007), cartilage was cut into pieces (approximately 1 mm^3) and digested with 10% collagenase type II (Sigma, USA) at 37 °C for 21 h. After that, cartilage pieces were washed in PBS (phosphate buffer saline) and incubated in a culture medium containing DMEM supplemented with 10% fetal bovine serum (FBS) (Gibco, USA) and 50 µg/ml gentamicin. Samples were cultured at 5% CO_2 and 37 °C. The medium was renewed twice a week. Primary chondrocytes migrated from cartilage in one to two weeks.

Bromelain treatment of cells. When the cells reached confluence, they were trypsinized (trypsin; Gibco, USA), counted and re-seeded in a 24-well plate at an initial density of 1×10^5 cells/well. After incubation for 48 h, cells were washed two times in PBS. Chondrocytes were then exposed to a high concentration of bromelain for different time periods (4, 16 and 32 h). Control groups for each exposure time were also created by treating media with an equal volume of DMEM instead of bromelain. Then, the six treatment groups (including the three control groups) were evaluated for morphological changes.

Morphological changes. All changes were evaluated after the conclusion of treatment. Staining was analyzed visually and captured by photographs under a phase contrast inverted microscope. Cell viability was tested by staining using a trypan blue (Gibco, USA) exclusion test. The percentages of stained cells (indicative of nonviable cells) versus stain-excluding cells were counted. Then the percentage of viable cells was calculated as follows: viable cell (%) = (total number of viable cells × 100)/total number of cells. The apoptotic rate was evaluated by staining with the vital DNA-binding dye Hoechst No. 33342 (Sigma). The reagents were diluted with 20% serum DMEM and incubated with the cells for 15 min before exposure to fluorescent light by an inverted fluorescence microscope. Dead cells were readily recognized, as they had a condensed or fragmented nucleus. Then the percentage of apoptotic cells was calculated as follows: apoptotic cell (%) = (total number of apoptotic cells × 100)/total number of cells. Additionally, the morphology of the cells was studied using acetoorcein dyes that can separate mitotic cells from interphase cells. Then, the percentage of mitotic cells was calculated as follows: mitotic index (%) = (total number of mitotic cells × 100)/total number of cells.

Glycosaminoglycan concentrations. One objective of this study was to investigate the values of glycosaminoglycan sulfate (S-GAG) and hyaluronan (HA), which can indicate biochemical alterations in articular cartilage, in the cultured media in each treatment group. Cultured media samples (0.5 ml) were collected in microcentrifuge tubes and stored at –20 °C until analysis.

Glycosaminoglycan sulfate (S-GAG) concentrations were determined using a colorimetric dye binding assay, modified according to the method of Pothacharoen (2002). The assay is based on a metachromatic shift in absorption maximum from 600 nm to 535 nm when a complex is formed between a mixture of 1,9-dimethylmethylene blue and the S-GAG in the culture media or standard. The dye solution was prepared by adding 16 mg of 1,9-dimethylmethylene blue dissolved in 5 ml ethanol to 2 g of sodium formate and 2 ml of formic acid in a total volume of 1 liter at pH 3.5. The maximum absorbance of the dye solution was at 590 nm. This solution was stored at 4 °C in a dark bottle. Chondroitin 6-sulfate (CS-C) standards (0–30 µg/ml: 50 µl) or samples (50 µl) were transferred to a microtitre plate. The dye solution (200 µl) was added immediately to each well, and the absorbance was measured at 590 nm (a precipitate will form after standing). A standard curve was plotted using the standard data, and then a sample's CS-C concentration was calculated from the standard curve.

Measurements of hyaluronan concentrations were performed using an enzyme-linked immunosorbent assay (ELISA), according to the method of Peansukmanee (2002). A 96-well microtitre plate was coated with umbilical cord HA coating buffer (100 µl/well) and left overnight at room temperature. Free sites were blocked with 1% (w/v) BSA in incubation buffer (100 µl/well; 0.01M Tris-HCl, 0.15M sodium chloride, 0.1% (v/v) Tween 20, pH 7.4) for 1 h at 25 °C. The plates were washed three times with incubation buffer. Then 100 µl of the mixture, media sample or standard competitor (HA Healon®: range 3.9–1000 ng/ml) in HA binding protein (b-HABP), were added. After incubation at 25 °C for 1 h, the plates were washed again, and then a peroxidase-mouse monoclonal anti-biotin (100 µl/well; 1 : 4000) was added. After incubation, the plates were washed as before, and then the peroxidase substrate (100 ml/well) was added. The plate was incubated at 37 °C for 10 min to allow the colour to develop. Absorbance was measured at 492/690 nm using a Titertek Multiskan M340 microplate reader. The HA concentration in the media sample was calculated relative to a standard curve generated from the standard competitor.

Cell collection. Cells were trypsinized using 0.25% trypsin/EDTA to produce a single-cell suspension. This was then aspirated into a centrifuge tube, and centrifuged at 1100–1200 rpm for 10 min at room temperature. Cell pellets were collected, and the supernatant discarded. Then the cell pellets were washed with PBS and centrifuged again under the same conditions.

RNA isolation and cDNA synthesis. Total RNA extraction was carried out using the RNeasy® Mini Kit (Qiagen, Germany) according to the manufacturer's instructions. The quality of RNA from each sample was assured by the A260/280 absorbance ratio and by electrophoresis in a 1% agarose gel in 1 × TAE buffer. Total RNA was reverse transcribed into single strand cDNA using M-MuLV reverse transcriptase (RT) (Vivantis, USA). The RT reaction was carried out for 5 min at 65 °C, followed by incubation at 37 °C for 5 min, and then at 42 °C for 90 min. The reaction was then inactivated by heating at 70 °C for 10 min in a thermocycler.

Primer design and real time PCR. All oligonucleotide primer sets were designed based on the published mRNA sequence. The expected amplicon lengths ranged from 70 to 200 bp. The oligonucleotide primers used in this study are listed in Table 1. Real-time PCR was performed in a SmartCycler II (Cepheid, Sunnyvale, CA) by using QuantiTect SYBR® Green PCR Master Mix

Table 1. Sequences of sense and antisense primers used for amplification in real-time PCR

Gene	Accession number	Primer sequence (5'→ 3')	Temperature (°C)	Amplicon size (bp)
AGG	U65989_2	Rw: ACTGCTCCAGGCGTGTGATG Fw: GACCATGTCGTGCAGGTGAC	58	405
COL2A1	AF023169	Rw: TGCTTTCCAGTTGGGCCAGC Fw: GAGCTCCTGGTGCATCTGGA	58	233
TIMP-1	AF077817_1	Rw:TGTCACTCTGCAGTTTGCAG Fw: GATGTTCAAGGGTTTCAGCG	55	294
MMP-3	AY183143_1	Rw: CAGAGCTTTCTCAATGGCAG Fw: CTCACCCAGCAATACCTAGA	55	297
GAPDH	DQ403060	Rw: CGAAGTGGTCATGGATGA CT Fw: AGT ATG ATT CTA CCC ACG GC	55	362

(Qiagen, Germany). Three replicates of 1.5 µl of the cDNA template were used for real-time PCR in a final volume of 20 µl. cDNA was amplified according to the following conditions: 95 °C for 15 s, and 60 °C for 60 s, from 35 to 45 amplification cycles. Changes in fluorescence were monitored with SYBR Green after every cycle. A melting curve analysis was performed (0.5 °C/s increasing from 55 to 95 °C with continuous fluorescence readings) at the end of the cycles to ensure that single PCR products were obtained. Amplicon size and reaction specificity were confirmed by 2.0% agarose gel electrophoresis. All reactions were repeated in three separate PCR runs using RNA isolated from three bio-replicates of the samples. The results were evaluated using the SmartCycler II software program. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression was used to normalize expression in the samples. To monitor crossover contamination of PCR, RNase-free water (Qiagen, Valencia, CA) was included in the RNA extraction, and was used as a negative control. To ensure quality of data, a negative control was always included in each run.

Statistical analysis. All data were analyzed using the statistical program SPSS, version 14. Student's *t*-test was performed for comparison of data obtained from the control and experimental groups by repeated measurement of analysis of variance. All data were expressed as means ± standard error. Differences were considered significant at $P < 0.05$. Tests were carried out at least in triplicate.

RESULTS

Effect of high doses of bromelain on cell morphology

Bromelain treatment (200 µg/ml to 2 mg/ml) of adherent canine chondrocyte cells resulted in chondrocytes remaining unattached to the plastic culture surface (Figure 1B, C). The efficiency depended on bromelain concentration. At high concentrations, a turbid sediment of bromelain was perceptible (Figure 1C, as indicated by arrows). Surprisingly, despite bromelain's ability to inhibit the adhesive force between cells, and between cells and a plastic surface, bromelain did not adversely affect cell viability, as determined by trypan blue dye exclusion – even at the highest dose level of 2 mg/ml (data not shown). Although bromelain did not cause cell death, bromelain did change cell attachment. That created difficulties in analyzing the morphological changes of the cells under a microscope, particularly during comparison with control groups whose cells were more adhesive. Therefore, we attempted to find the appropriate bromelain concentration (1, 20 or 50 mg/ml and 500 ng/ml) to treat chondrocytes in order to prevent dependence of the cells. Since none of the bromelain treatments were found to be independent of the cells (data not shown), this study used the highest concentration of 50 mg/ml to assess the effect of bromelain on canine chondrocytes. Six groups of treatment were designed, which received bromelain for 4, 16

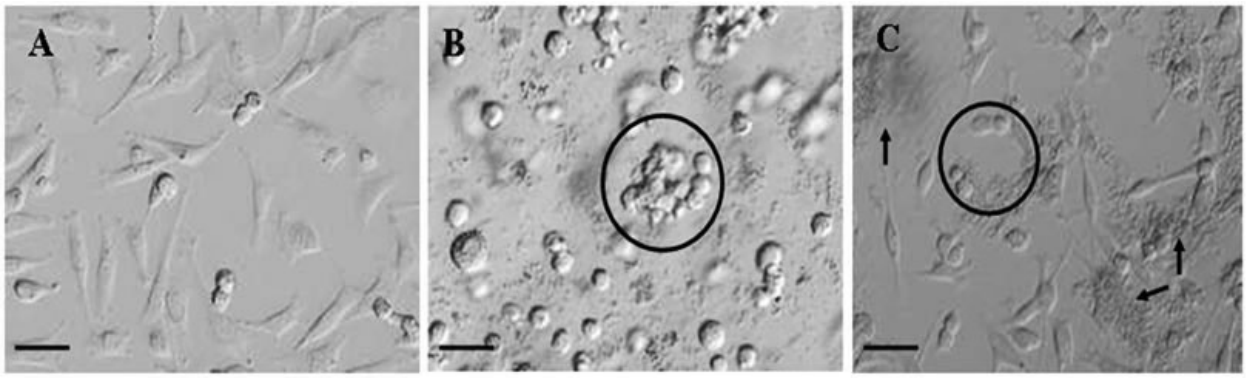

Figure 1. Chondrocyte morphogenesis during bromelain treatment. (A) Chondrocyte morphology in the control groups. (B) Chondrocyte morphogenesis during exposure to 2 mg/ml of bromelain dependent suspension of the cells and colony of cell suspension (in circle). (C) Chondrocyte morphogenesis during exposure to 200 µg/ml of bromelain. The cells showed a characteristically round cytoplasm but had not lifted from the surface (in circle). Bromelain sediment is indicated by arrow. Size bar = 30 mm

Table 2. Comparison of viability, apoptotic rate and mitotic index between the bromelain-treated group (T) and the control group (C) (without treatment) at different bromelain exposures

Duration (hours)	Group	Viability rate (%)	Apoptotic rate (%)	Mitotic rate (%)
4	T	99.10 ± 0.37	2.21 ± 0.67	11.51 ± 0.67*
	C	99.55 ± 0.18	3.54 ± 0.46	5.44 ± 0.46
16	T	98.90 ± 0.33	2.45 ± 0.78*	13.39 ± 0.78*
	C	99.39 ± 0.25	4.50 ± 0.62	5.70 ± 0.63
32	T	98.40 ± 0.63	1.62 ± 0.46*	12.78 ± 0.46*
	C	98.20 ± 0.50	3.90 ± 0.40	4.76 ± 0.40

A significant difference ($P < 0.05$) between treatment and non-treatment with bromelain for the same period is displayed with superscript (*) on the number

and 32 h. Control groups were cultured in medium alone for the same exposure times.

Cell viability by the trypan blue dye exclusion method

Chondrocytes were stained with trypan blue dye and photographed. The pictures showed viable (unstained) cells, and dead (stained) cells which had lost plasma membrane integrity. Dead cells showed a blue stain throughout the whole cell (data not shown). The calculated mean of cell viability in each group (Table 2) revealed that no significant differences were detected between the bromelain treatment groups and the control groups at all duration times. These results indicated that 50 mg/ml of bromelain did not affect the viability of chondrocytes.

Effect of bromelain on cell apoptosis by the Hoechst exclusion method

The DNA content of chondrocytes after apoptosis was determined using Hoechst dye No.33342; cells were photographed under a fluorescence microscope. Apoptotic cells may have condensed DNA. When stained with dye, these cells extend radially for a greater distance than do non-apoptotic cells (data not shown). The calculated mean of apoptosis percentage in each group (Table 2) shows that the percentages of apoptosis in all bromelain-treated groups (at all exposure times) were lower than in the control groups. Compared to the control groups, the chondrocytes in bromelain-treated groups had significant reductions in the percentage of apoptosis ($P < 0.05$) at both 16 and 32 h of bromelain exposure. Thus, bromelain was clearly shown to modulate the apoptosis of chondrocyte cells.

Mitotic index using aceto-orcein dyes

Bromelain-exposed groups and control groups were subjected to aceto-orcein staining to detect mitotic cells. After counting and calculating raw data (Table 2), it was found that the mean mitotic indexes in bromelain treatment groups were significantly ($P < 0.05$) higher than in control groups at all time exposures. These data indicated that bromelain could increase chondrocyte cell proliferation.

Glycosaminoglycan concentrations

Two kinds of GAG, S-GAG and HA, were obtained from culture media after incubation of cells for 48 h. Then the absorbance values of each group were compared with a standard curve. As indicated in Table 3, there was an increase in all S-GAG and HA levels after 32 h of bromelain exposure. This suggested that glycosaminoglycan concentrations were dependent on the duration of bromelain exposure, with the longest exposed time being 32 h. Although glycosaminoglycan synthesis increased after chondrocytes were exposed to bromelain, glycosaminoglycan levels in all treatment groups were not significantly different from their control groups.

Table 3. Glycosaminoglycan sulfate (S-GAG) and hyaluronan (HA) levels in six groups comparing the bromelain-treated group (T) and the control group (C) (without treatment) at different bromelain exposures

Duration (h)	Group	S-GAG (μg/ml)	HA (μg/ml)
4	T	13.77 ± 0.69	13.20 ± 1.48
	C	14.65 ± 3.05	11.45 ± 0.55
16	T	13.63 ± 0.43	11.72 ± 1.25
	C	14.85 ± 1.65	16.45 ± 4.45
32	T	15.78 ± 0.88	27.60 ± 2.89
	C	13.80 ± 1.00	18.40 ± 1.50

Effect of bromelain on gene expression

Chondrocytes in treatment groups were exposed to 50 μl/ml bromelain, whereas the control groups were exposed to DMEM only. After the treatment, the cells were incubated with DMEM + 10% serum for 48 h; then total mRNAs were extracted from chondrocytes. The relative expression levels of mRNAs were assessed by real-time PCR, and results were normalized to GAPDH. We examined whether there were alterations in the expression patterns of four genes – aggrecan (AGG), collagen type II (COL2A1), matrix metalloproteinase-3 (MMP-3), and tissue inhibitor of metalloproteinases (TIMPs) – to confirm the phenotypic characteristics and degradative enzyme production of

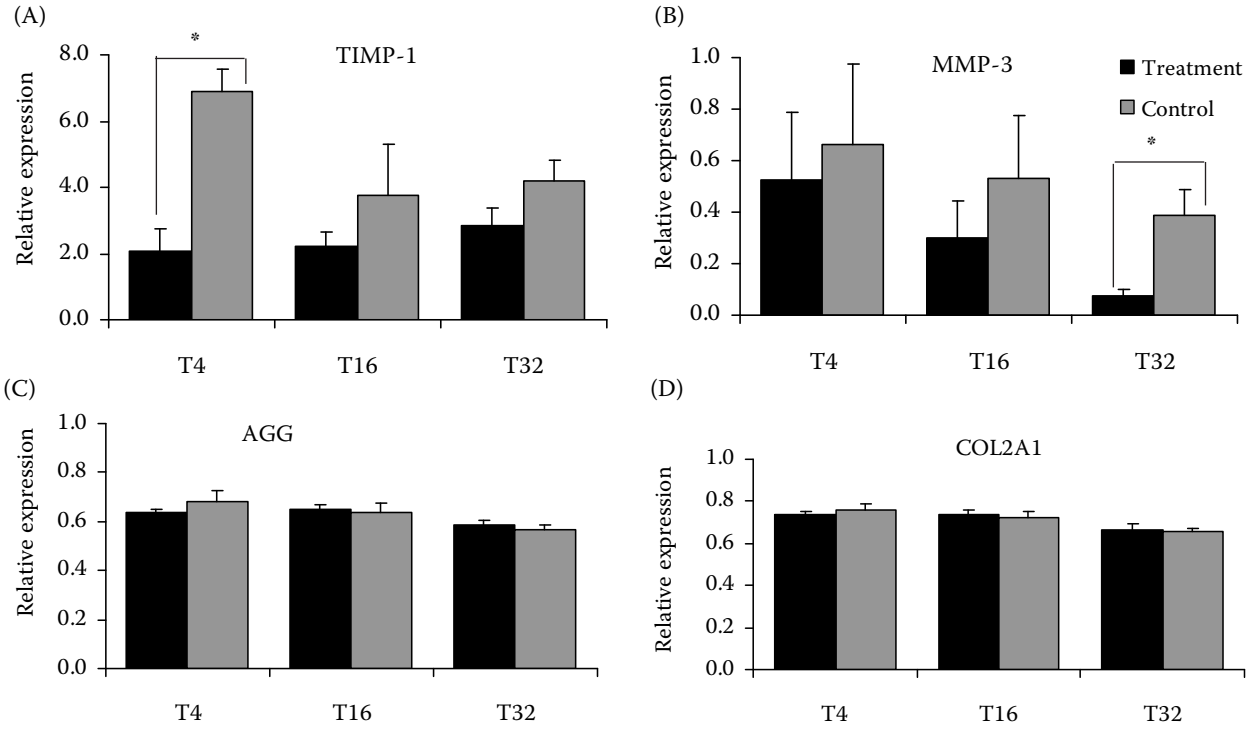

Figure 2. The effects of 50 μg/ml bromelain treatment on the relative expression of mRNAs were assessed by real-time PCR and results were normalized to GAPDH. (A) The mRNA expression levels of TIMP-1 were decreased in bromelain-treated groups compared with control groups. (B) The mRNA expression levels of MMP-3 in bromelain-treated groups were decreased compared with control groups. (C, D) The expression of mRNA coding for two ECM molecules AGG and COL2A1 compared with control groups. Bromelain exposure times were 4, 16 and 32 h. Data represent mean ± SE (triplicated culture representative of n = 3 experiments performed)

*$P < 0.05$ using paired t-test

chondrocytes (Figure 2). There was a significantly decreased TIMP-1 expression after 4 h of bromelain treatment (2.05 ± 0.68), compared to the control group (6.89 ± 1.53) at the same exposure time. Groups at other exposure times showed similarly decreased levels of TIMP-1 when compared to the control groups, but the differences were not significant (Figure 2). The mRNA expression levels of MMP-3 presented a decreased tendency and had a negative correlation with exposure times. At 32 h of bromelain exposure, MMP-3 mRNA expression was significantly decreased relative to the control group (Figure 2). We next examined the expression of the mRNAs coding for two extracellular matrix (ECM) component molecules, AGG and COL2A1 (Figure 2), compared with the control groups. The expression of AGG and COL2A1 did not significantly change. To understand the effect of bromelain in curing OA, we concentrated on its control of the activity of MMPs. This appeared to lie in the apparent imbalance between MMPs and TIMPs that are produced by chondrocytes (Denko and Malemud, 1999), where MMP gene expression is markedly elevated at the expense of TIMP. The results of this study indicated that although bromelain decreased the mRNA expression of TIMP-1 and MMP-3, it did not decrease the expression of AGG and COL2A1.

DISCUSSION

This study is the first to demonstrate the direct effect of bromelain on canine chondrocytes *in vitro*. The characteristics of the primary chondrocyte in this study were most similar to those used by Yang et al. (2000) and Frohlich et al. (2007). The process of isolation from cartilage tissue is an important step, because at this point it must be ensured that there are sufficient viable cells for further cultivation and analysis. The limited degree of cell proliferation and the accompanying dedifferentiation during culture make it essential to obtain the maximum yield of cells from a biopsy of limited size. Because cells were collected from non-OA joints in canine articular cartilage obtained from young dogs (aged three to five years old and weighing about 10–20 kg), and were isolated and cultured for short periods of time, our primary cells had many characteristics of chondrocytes embedded in an abundant extracellular matrix

In experiments with high concentrations (200 µg/ml to 2 mg/ml) of bromelain, it was found that the independence of cells and their efficiency depended on bromelain concentrations (Figure 1B, C). Bromelain inhibited the adhesive force between cells, and between cells and a plastic surface; but bromelain did not adversely affect cell viability. This indicated that bromelain has effects similar to the enzyme proteinase. Other experiments have previously shown that bromelain proteolytically removes certain cell surface molecules by cleavage of the peptide bond (Hale and Haynes, 1992; Maurer, 2001; Barth et al., 2005). It has been shown that bromelain can inhibit some intracellular signalling pathways and reduce cell surface adhesion molecules. Bromelain has also has been shown to reduce cell surface receptors such as the hyaluronan receptor CD 44, which is associated with leukocyte migration (Hale et al., 2002). It was clearly demonstrated that bromelain at high concentrations does not adversely affect cell viability; similarly, a bromelain concentration of 50 mg/ml had no effect on the viability of chondrocytes. Our results comply with previous reports on the viability of cells after bromelain treatment, wherein bromelain offers a wide spectrum of efficacies but does not initiate cell death (Hale and Haynes, 1992; Mynott et al., 1999; Engwerda et al., 2001).

We found that bromelain modulated the apoptosis of chondrocyte cells and increased chondrocyte cell proliferation, as evaluated by the mitotic index. Apoptosis has been identified as one mechanism of cell death. Reduced cellularity is believed to contribute to the pathogenesis of OA. Studies using electron microscopy and fluorescent staining of OA cartilage have demonstrated the presence of cellular fragmentation and nuclear condensation. Apoptotic bodies have similar structures to matrix vesicles, and they may promote the calcification of pathologic cartilage (Blanco, 1999; Nesic et al., 2006). Reduced apoptotic rates and enhanced cell proliferation following bromelain treatment have been reported (Mynott et al., 1999; Barth et al., 2005), showing that bromelain acts as a signalling molecule which inhibits Raf-1/extracellular-regulated kinase (ERK)-2 transduction to from participating in apoptosis, mitogenesis and cytokine production. It is evident that bromelain exhibits an immunomodulatory effect, resulting in enhanced cell proliferation activity. However, bromelain has been shown to have an adverse effect on tumour cells by upregulating p53 and Bax, decreasing levels of the anti-apoptotic protein Bcl-2 in mouse skin, significantly inhibiting cyclooxygenase-2 (COX-2),

and inactivating NF-κB. This resulted in apoptosis of tumour cells (Arora et al., 2004; Kalra et al., 2008). In OA the apoptosis of chondrocyte cells involves the activity of nitric oxide (NO), inducible nitric oxide synthase (iNOS), COX-2, and prostaglandin E2 (PGE2) (Blanco, 1999; Goldring, 2000; Sandell and Aigner, 2001; Nesic et al., 2006). From our preliminary studies, we suspect that because bromelain exerts an inhibitory effect on NO, iNOS, COX-2 and PGE2 (Oh-ishi et al., 1979; Wen et al., 2006; Kalra et al., 2008), bromelain significantly decreases the rate of apoptosis and increases chondrocyte proliferation. Following from these results it will be interesting to elucidate the different actions of bromelain treatment on further cellular targets.

We found that a longer duration of bromelain treatment correlated with increased glycosaminoglycan concentrations; S-GAG and HA from chondrocytes in the culture medium, including the upregulation of mRNA levels corresponding to matrix markers; and increased gene expression of AGG and COL2A1, consistent with earlier reports (Rovenska et al., 1999, 2001). However, the results were not significantly different from the control, and this effect was only observed when cells were treated with bromelain concentrations of 50 mm/ml or less. Nevertheless, there was a different difference between the effect of bromelain versus other proteinases such as collagenase, cathepsin or papain, which had an adverse effect on the cartilage matrix component and matrix synthesis (van Osch et al., 1998; Yang et al., 2000; Cawston and Wilson, 2006) while bromelain showed no harmful effects. It is suspected that bromelain contains several distinct cysteine proteinases that have distinct amino acid sequences, as well as differences in proteolytic specificity and sensitivity to inactivation (Hale et al., 2005; Salas et al., 2008).

This result is in agreement with previously reported studies, which showed that bromelain diminishes MMP expression (Maurer, 2001 and Tochi et al., 2008). This is consistent with our result, which found decreased expression of MMP-3 mRNA levels in a manner dependent on the duration of bromelain exposure. MMP-3 is an enzyme which cleaves the non-helical telopeptide of type II and type IX collagens, leading to the presence of OA (Goldring, 2000; Sandell and Aigner, 2001). However, this does not explain the effect of bromelain on the evident decrease in TIMP-1 and MMP-3 levels. Therefore, it has been hypothesized that the exogenous influence of bromelain can proteolytically remove certain cell surface molecules, as described previously (Hale and Haynes, 1992; Maurer, 2001; Barth et al., 2005). This can cause the alteration in MMP and TIMP synthesis controlled by the transmembrane domain at the chondrocyte cell membrane (Cawston and Wilson, 2006). All active MMPs are inhibited by TIMPs that bind tightly to active MMPs in a 1 : 1 ratio in normal chondrocytes (Denko and Malemud, 1999; Cawston and Wilson, 2006).

In summary, all the results in the present study point to a promising future for the use of bromelain in the treatment of OA. However, bromelain is comprised of an unusually complex mixture of different thiol endopeptidases and other not-yet-completely characterized components, such as phosphatases, glucosidases, peroxidases, cellulases, glycoproteins and carbohydrates, among others (Rowan and Buttle, 1994; Maurer, 2001). In addition, bromelain contains several proteinase inhibitors (Maurer, 2001). Thus it is difficult to be sure of the effect of bromelain on chondrocytes. Although this research established that bromelain can control degradative processes and produce some glycosaminoglycans, further evaluation is needed before definitive conclusions can be made regarding the use of bromelain in OA treatment.

The results of the present study show that 50 μg/ml of bromelain can decrease the apoptotic rate in canine chondrocytes, and is able to significantly activate the proliferation of these cells while not adversely affecting their viability. Furthermore, the results show a statistically significant decrease in the relative expression of TIMP-1 and MMP-3 mRNAs in bromelain-treated groups compared to the control groups. The effect of bromelain on chondrocytes was found to be strongly dependent upon bromelain concentration. It was found that 2 mg/ml to 200 μg/ml could prevent chondrocytes from adhering to the plastic culture surface, but did not affect cell viability.

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