CHAPTER III

RESULTS

3.1 Investigation of chondroprotective effect of *Alpinia galanga* extracts and screening for active phytochemical

3.1.1 Chondroprotective effect of hexane, acetone, ethylacetate, and methanol extracts of *Alpinia galanga* in porcine cartilage explant induced inflammation using IL-1β

To investigate the inhibitory effects of *A. galanga* rhizome (Figure 3.1) extracts on the degradation of the extracellular matrix (ECM) in cartilage, porcine cartilage explants were co-treated with proinflammatory cytokine IL-1β (10 ng/ml), and various concentrations of the extracts (6.25, 12.5 and 25 μg/ml). After treatment for 3 days, the conditional media were collected and analyzed. Dye-binding assay, ELISA and gelatin zymography were used to determine levels of s-GAG release, HA release and MMP-2 activity, respectively. Uronic acid remaining in cartilage was also determined. The conditioned cartilage discs were digested with papain and then uronic acid content was measured.

Figure 3.1 Characteristic of *Alpinia galanga* (left panel) and it's rhizome (right panel)(http://botany.csdl.tamu.edu/FLORA/imaxxzin.htm).

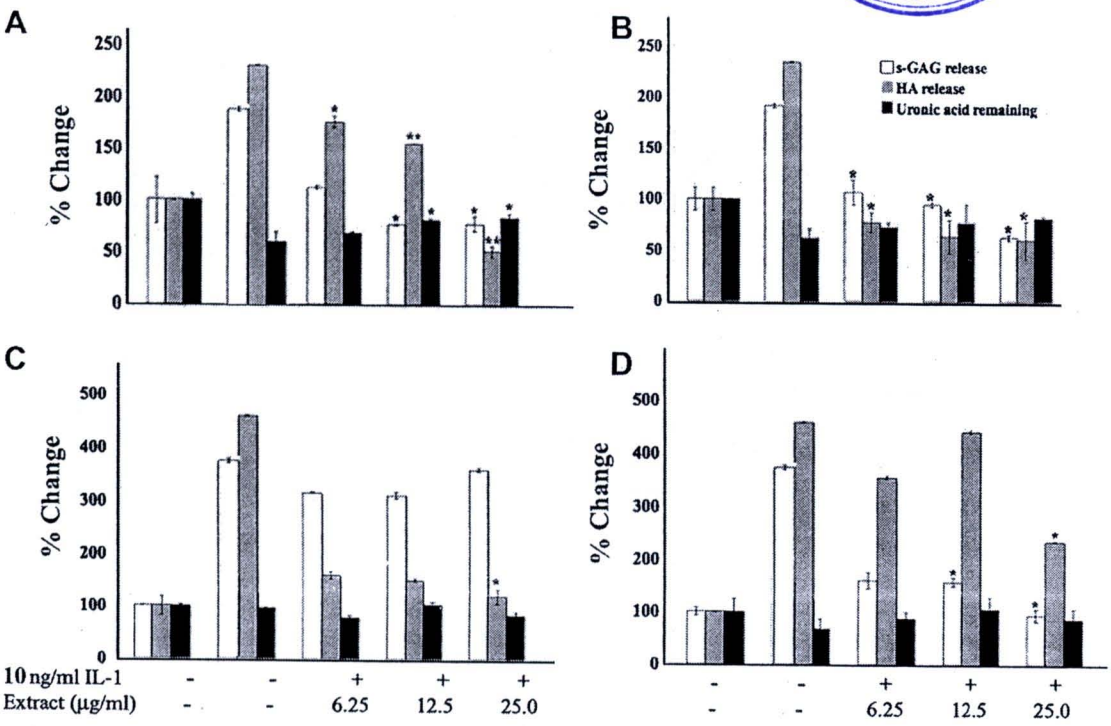

Figure 3.2 *A. galanga* extracts affected on the releases of s-GAG, HA from porcine cartilage tissue to the media and uronic acid remaining in the cartilage tissue. Cartilage explants were cultured with IL-1β (10 ng/ml) in absence and presence of *A. galanga* hexane (A), acetone (B), ethylacetate (C), and methanol (D) extracts (at concentrations of 6.25 - 25 µg/ml) for 3 day. In the media, s-GAG, HA releases were measured using dye-binding assay and ELISA respectively. Cartilage discs were digested with papain and then the uronic acid content was measured.

* and ** denote a value that is significantly different ($p<0.05$ and $p<0.01$, respectively) from the IL-1β control.

As shown in figure 3.2, it was found that IL-1β alone increased s-GAG and HA releases from cartilage to media. Co-cultured with of IL-1β and the individual of hexane, acetone, ethylacetate and methanol extract, significantly reduced the levels of s-GAG release. Anyhow, the release of HA from the explants treated with IL-1β was significantly reduced only by the acetone and hexane fractions. The hexane and acetone fractions inhibited reduction of uronic acid in cartilage tissue that was induced by IL-1β (figure 3.2), whereas the ethylacetate and methanol extracts did not.

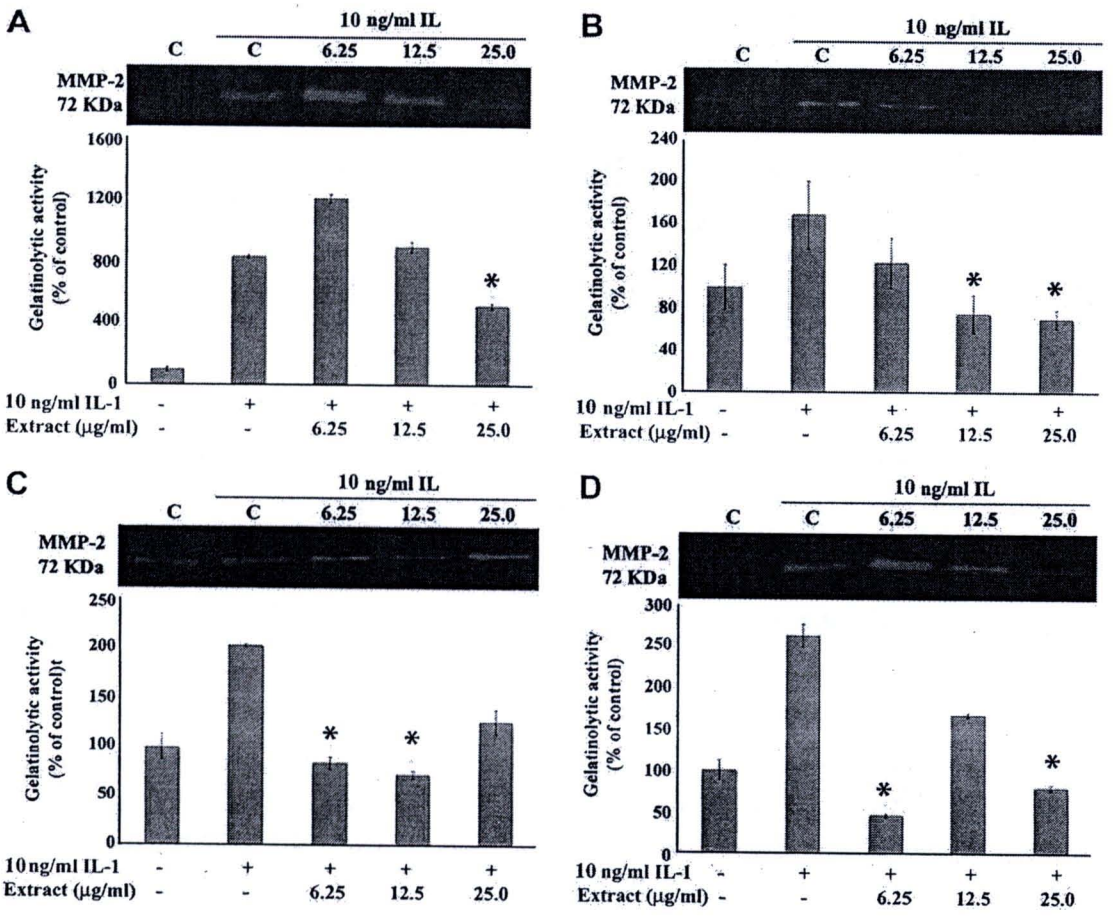

Figure 3.3 Effects of *A. galanga* extracts on MMP-2 activity. Porcine cartilage explants were cultured with IL-1β (10 ng/ml) in the absence and presence of *A.galanga* hexane (A), acetone (B), ethylacetate (C) and methanol (D) extracts (at concentrations of 6.25-25 µg/ml) for 3 days. Media were collected and then analyzed by gelatin zymography as described in chapter II.

* and ** denote a value that is significantly different ($p<0.05$ and $p<0.01$, respectively) from the IL-1β control.

Figure 3.3 demonstrates that the activity of MMP-2 in the conditional media was found high level of pro-MMP-2 production in IL-1β induced cartilage. Furthermore, MMP-2 activity levels decreased under conditions of co-treatment between IL-1β and all four *A. galanga* extracts when compared with IL-1β treatment alone. However, DMSO used to dissolve the sample did not affect these ECM molecules releases (data not shown).

Among four fractions, it was found that acetone extract had the strongest activity, which could inhibit up to 90 % of IL-1β-induced-s-GAG release (at concentration 25 μg/ml) and up to 80 % of HA release (at concentration 12.5 μg/ml).

3.1.2 The isolation of active compound in acetone extract of *A. galanga*

From section 3.1.1, the acetone fraction had the highest chondroprotective effect. Column chromatography of this fraction resulted in many acetone sub-fractions (F1-F7, as shown in chapter II in the topic of isolation of active compound of acetone fraction of *A. galanga*). These sub-fractions were investigated for chondroprotective property using porcine cartilage explant to pick up the active sub-fraction. Fraction F5 showed the highest chondroprotective effect but this fraction was not pure (it showed smear band in TLC). Thus, the re-chromatography was performed and resulted other 3 sub-sub-fractions (A1-A3). Fraction A1-A3 was investigated for chondroprotective effect, A2 fraction was found as the active fraction. Similar to F5, fraction A2 showed smear band in TLC. Fraction A2 was re-separated using column chromatography and resulted other 3 sub-sub-sub-fractions (B1-B3). It was found that B2 fraction was the active fraction and showed the highest chondroprotective effect in porcine cartilage explant when compared with other two

fractions (B1 and B3). This fraction showed single spot in many TLC, this result suggested that B3 fraction was pure. The identification of this fraction was performed using MS/NMR spectroscopic analyses and revealed that the active phytochemical in this fraction was *p*-hydroxycinnamaldehyde (the structure of this phytochemical is shown in figure 3.4).

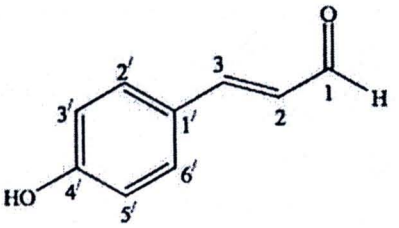

Figure 3.4 Para-hydroxycinnamaldehyde (1, 3- (4-hydroxy-phenyl)-propenal) the active compound of the acetone fraction of *A. galanga*.

3.1.3 The effect of *p*-hydroxycinnamaldehyde on porcine cartilage explant

The chondroprotective effect of active phytochemical of acetone fraction of *A. galanga* was shown in figure 3.5. It inhibited the effects of IL-1β on s-GAG, HA releases, MMP-2 activity levels, and the remaining of uronic acid in cartilage tissue.

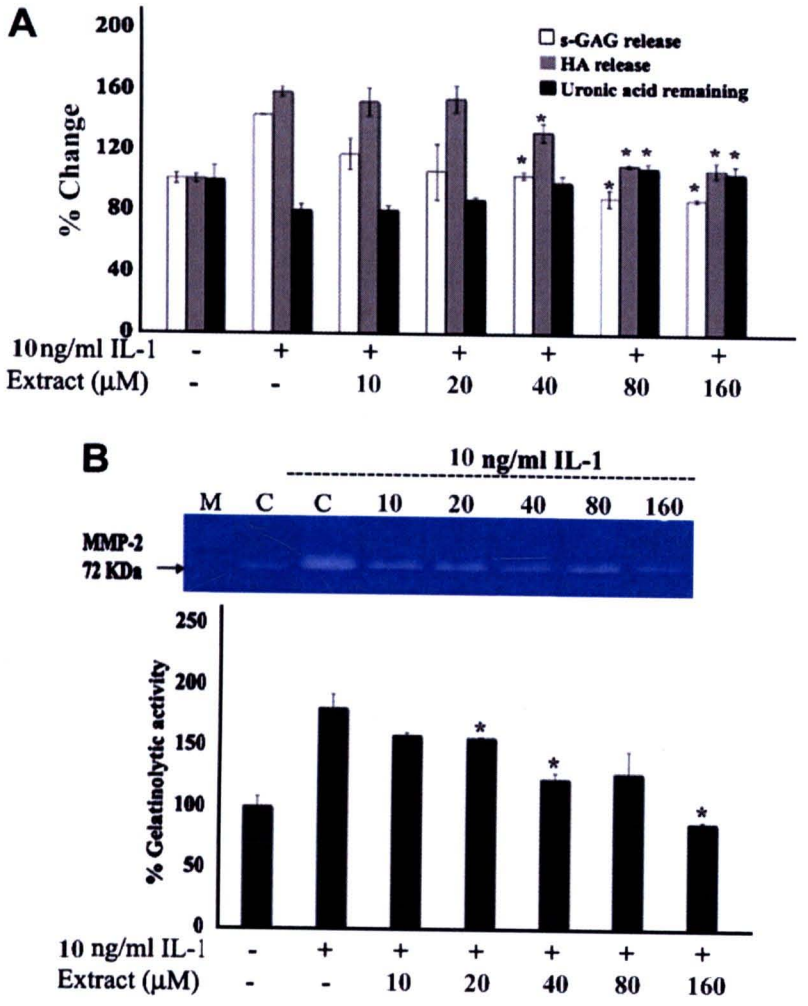

Figure 3.5 Para-hydroxycinnamaldehyde affect on the releases of s-GAG, HA from porcine cartilage explant to the media and the uronic acid remaining in the cartilage tissue (A) and the production of MMP-2 activity (B). Porcine cartilage explants were cultured with IL-1β (10 ng/ml) in the absence and presence of *p*-hydroxycinnamaldehyde for 3 days. S-GAG, HA and MMP-2 activity were measured in conditioned media and uronic acid remaining was performed in papain-digested cartilage tissue.

* denote a value that is significantly different ($p<0.05$) from the IL-1β control.

3.2 Investigation of chondroprotective effect of sesamin isolated from *Sesamum indicum* Linn.

3.2.1 Chondroprotective effect of sesamin in porcine cartilage explant induced inflammation using IL-1β

As described above that sesamin was reported the an-inflammatory effect. Figure 3.7 showed the chemical structure of sesamin. Porcine cartilage explant was performed for screening chondroprotective effect of sesamin. Thirty to 35 mg cartilage discs were cultured in 24-well plate and then co-treated with 10 ng/ml IL-1β to induce inflammation and various concentrations of sesamin for 3 days. HA, s-GAG and MMP-2 releases were measured in conditioned media. IL-1β could increase all HA, s-GAG and MMP-2 releases from cartilage to media, and sesamin had ability to revert these effects of IL-1β in the dose dependent manner (Figure 3.8 (A)). For uronic acid remaining in cartilage disc, after media had been collected, dried cartilage tissues were digested with papain and then measured uronic acid remaining using colorimetric method. It was found that IL-1β reduced uronic acid remaining in cartilage tissues and this reduction was reverted by sesamin in the dose dependent manner (Figure 3.8 (B)). These results suggested that sesamin had chondroprotective effects in porcine cartilage explant model.

Figure 3.6 Characteristic of *Sesamum indicum* (left panel) and sesame seeds (right panel) (http://www.bikudo.com/product_search/details/156707/sesamin_sesame_seed_lignan.html).

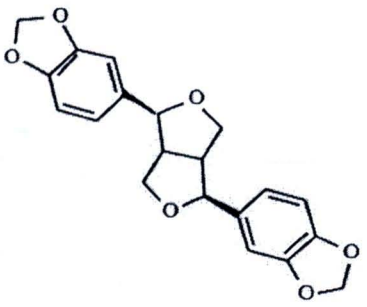

Figure 3.7 Sesamin: the phytochemical of the *Sesamum indicum.*

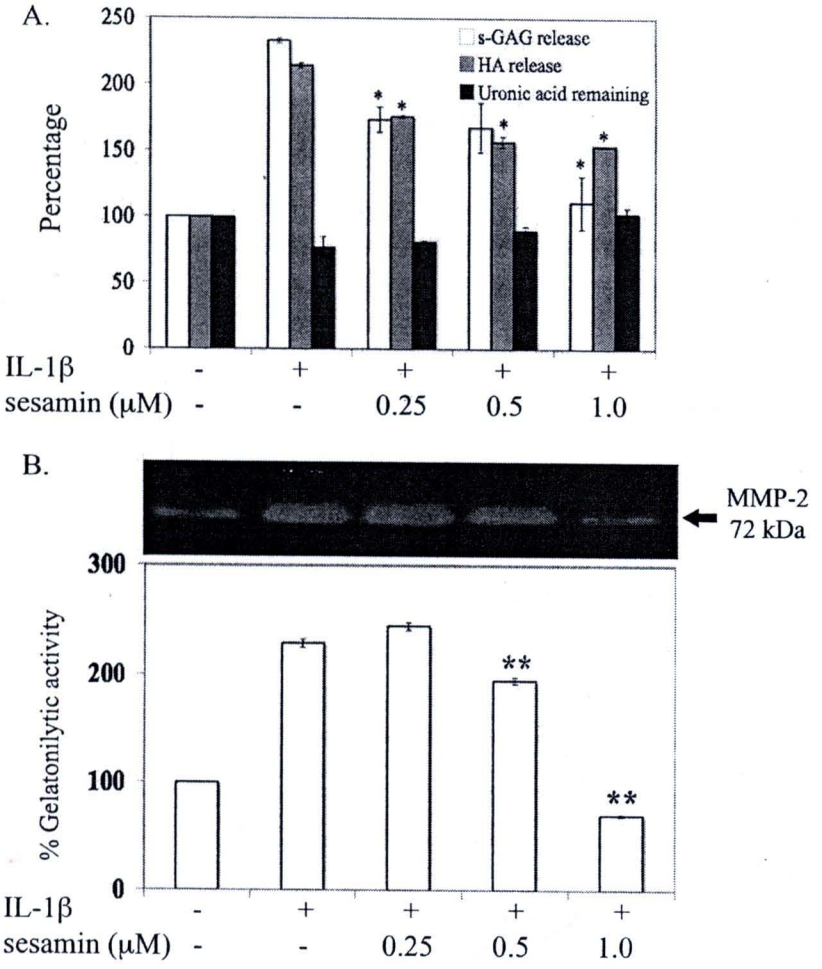

Figure 3.8 Effect of sesamin on the releases of s-GAG, HA from porcine cartilage explant to the media and the uronic acid remaining in the cartilage tissue (A) and the production of MMP-2 activity (B). Porcine cartilage explants were cultured with IL-1β (10 ng/ml) in the absence and presence of sesamin for 3 days. S-GAG, HA and MMP-2 activity were measured in conditioned media and uronic acid remaining was performed in papain-digested cartilage tissue.

* and ** denote a value that is significantly different ($p<0.05$ and $p<0.01$, respectively) from the IL-1β control.

3.3 Investigation of molecular mechanism of chondroprotective effect of *p*-hydroxycinnamaldehyde and sesamin in human articular chondrocytes (HACs)

3.3.1 Cytotoxicities of *p*-hydroxycinnamaldehyde and sesamin in HACs

Prior to investigate the molecular mechanisms of *p*-hydroxycinnamaldehyde and sesamin in HACs, the cytotoxics of both phytochemicals were investigated. From MTT assay, both effective doses of *p*-hydroxycinnamaldehyde (at the concentrations of 20-160 μM) and sesamin (at the concentrations of 0.25-1.00 μM) were not toxic to HACs. As shown in figure 3.9, the cell survival in the present of both phytochemicals did not lower than 80% when compared with control untreated cells.

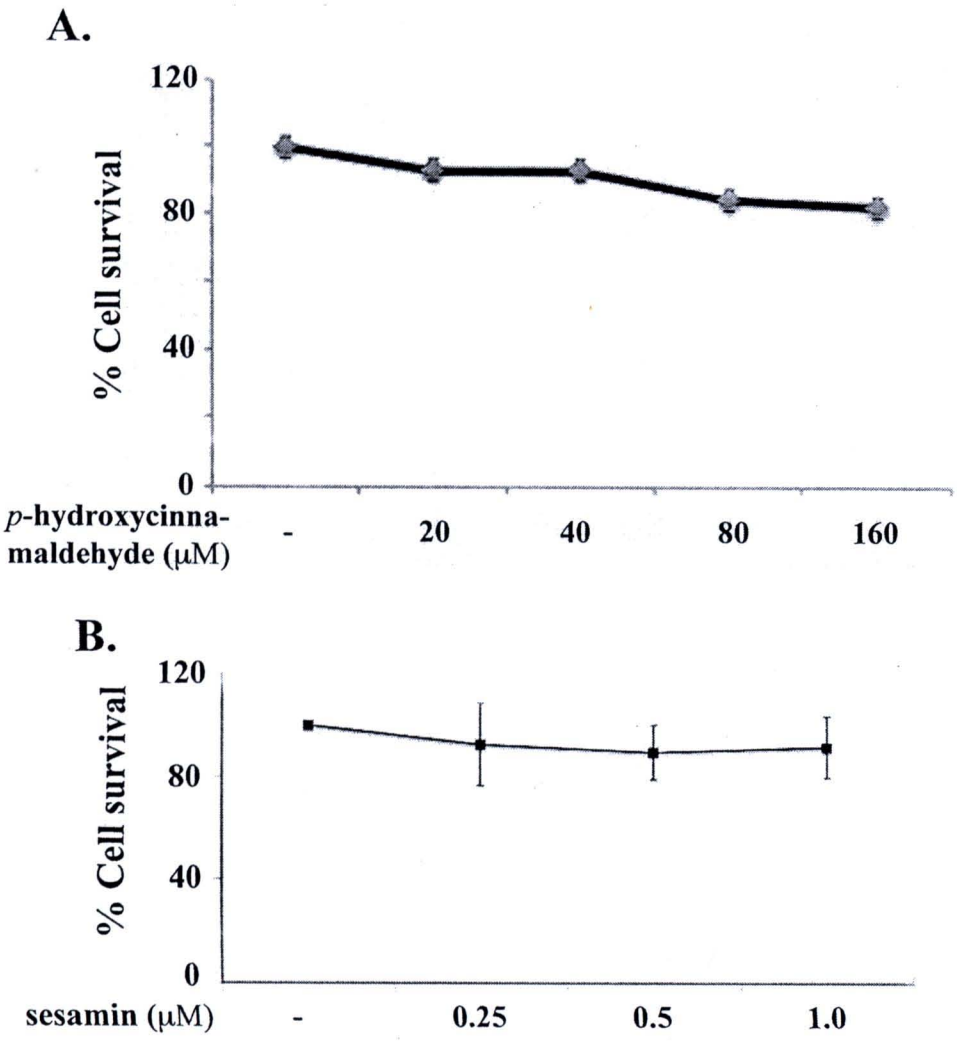

Figure 3.9 Cytotoxicities of *p*-hydroxycinnamaldehyde (A) and sesamin (B) in human articular chondrocytes measuring by MTT assay.

3.3.2 The effect of *p*-hydroxycinnamaldehyde and sesamin on human articular chondrocytes

We further determined the anti-inflammatory effect of *p*-hydroxycinnamaldehyde and sesamin using an IL-1β induced primary HACs monolayer culture model. As shown in figure 3.10 and 3.11, 10 ng/ml IL-1β induced releases of s-GAG, HA and MMP-2 into media. These inductions were significantly suppressed by *p*-hydroxycinnamaldehyde (figure 3.10) and sesamin (figure 3.11).

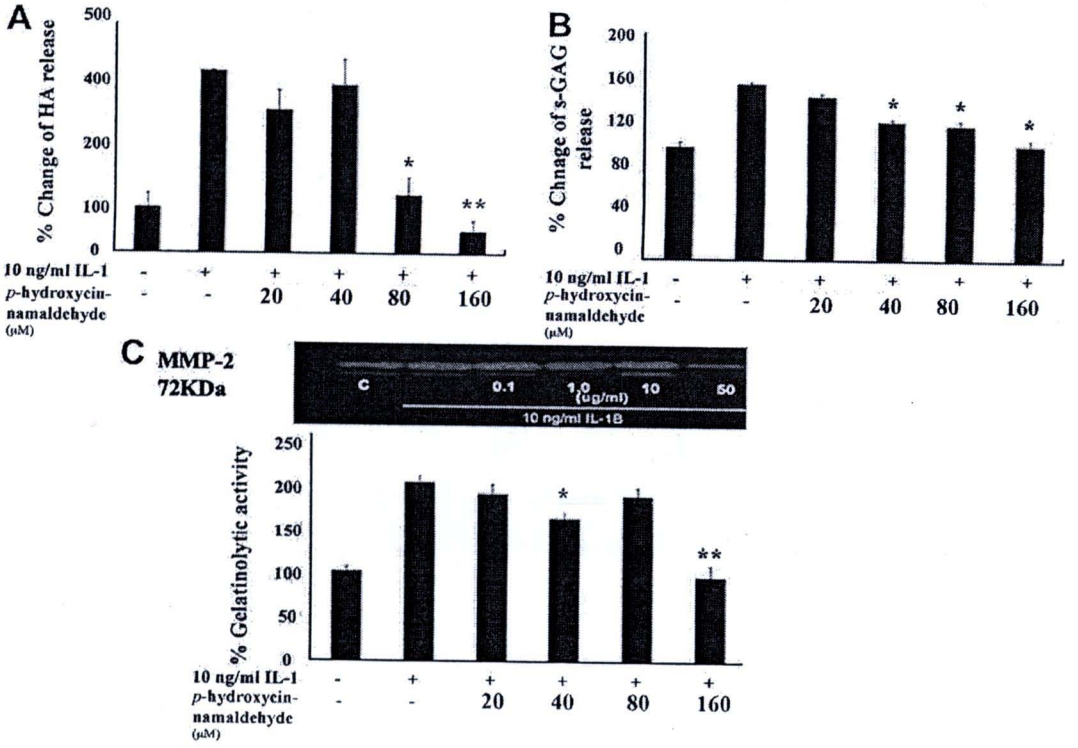

Figure 3.10 Effects of *p*-hydroxycinnamaldehyde on releases of HA (A), s-GAG (B) and MMP-2 (C) from chondrocytes. Chondrocytes were co-treated with 10 ng/ml IL-1β and various concentrations of *p*-hydroxycinnamaldehyde for 24 h. The conditioned media were analyzed for HA, s-GAG and MMP-2 activity.

* and ** denote a value that is significantly different ($p < 0.05$ and $p < 0.01$, respectively) from the IL-1β control.

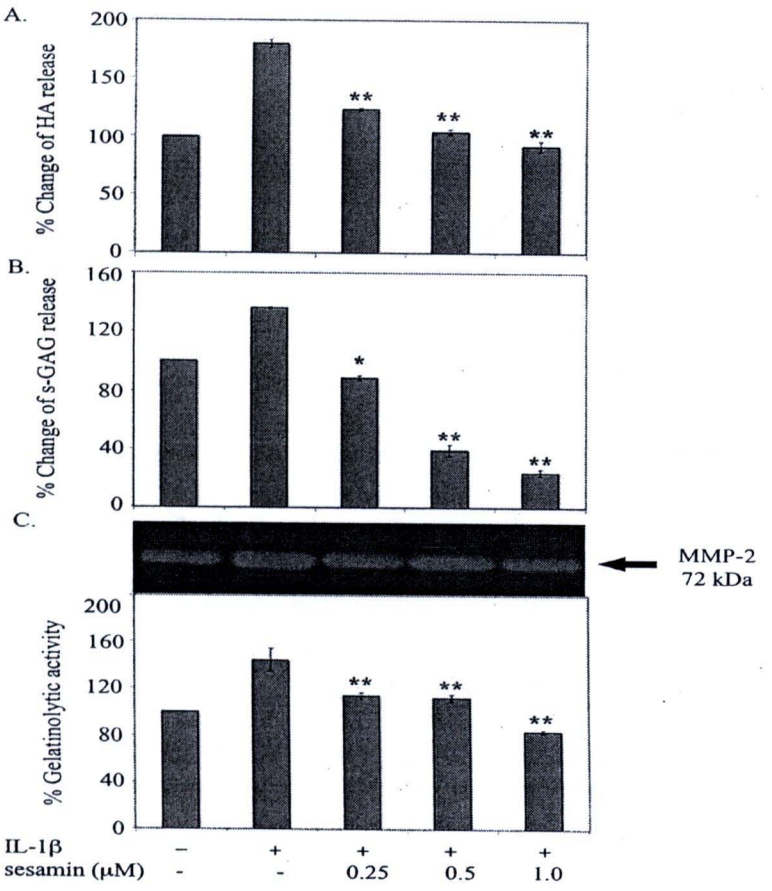

Figure 3.11 Effects of sesamin on releases of HA (A), s-GAG (B) and MMP-2 (C) from chondrocytes. Chondrocytes were co-treated with 10 ng/ml IL-1β and various concentrations of sesamin for 24 h. The conditioned media were analyzed for HA, s-GAG and MMP-2 activity.

* and ** denote a value that is significantly different ($p < 0.05$ and $p < 0.01$, respectively) from the IL-1β control.

3.3.3 The effect of *p*-hydroxycinnamaldehyde and sesamin on catabolic and anabolic gene expressions in HACs

Cartilage matrix maintains a balance between biosynthesis and degradation of ECM molecules, but IL-1β can disrupt this balance (244, 245). Effects of *p*-hydroxycinnamaldehyde and sesamin on gene expressions in the primary HAC were investigated using RT-PCR. The increased of the expression levels were shown in 10 ng/ml IL-1β induced the catabolic genes MMP-1, MMP-3 and MMP-13 (figure 3.12 and 3.13). Furthermore, it was shown that *p*-hydroxycinnamaldehyde could inhibit increased MMP-3 and MMP-13 gene expressions; by contrast, it did not affect levels of MMP-1 gene expression (figure 3.12). Whereas sesamin could inhibit increased MMP-1 and MMP-13 in the dose dependent manner, but did not for MMP-3 (figure 3.13).

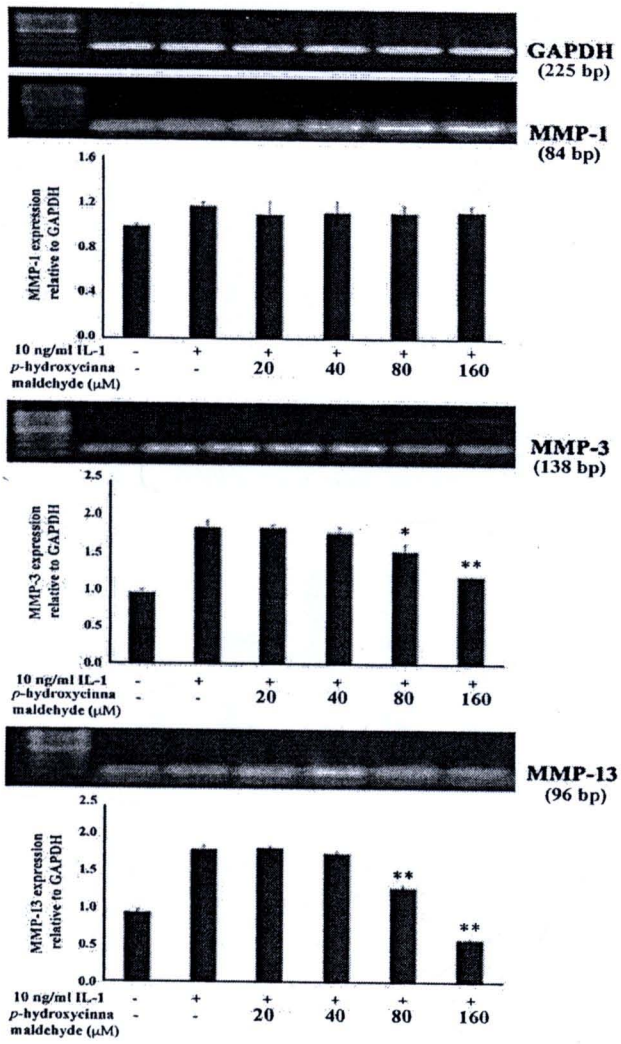

Figure 3.12 Effect of *p*-hydroxycinnamaldehyde on mRNA expression of proteinases (MMP-1, -3, -13). Confluent human chondrocytes in 25-cm^3 flasks were cultured with IL-1β (10 ng/ml) in the presence and absence of *p*-hydroxycinnamaldehyde for 24 h. Cells were harvested and gene expressions were analyzed.

* and ** denote a value that is significantly different ($p < 0.05$ and $p < 0.01$, respectively) from the IL-1β control.

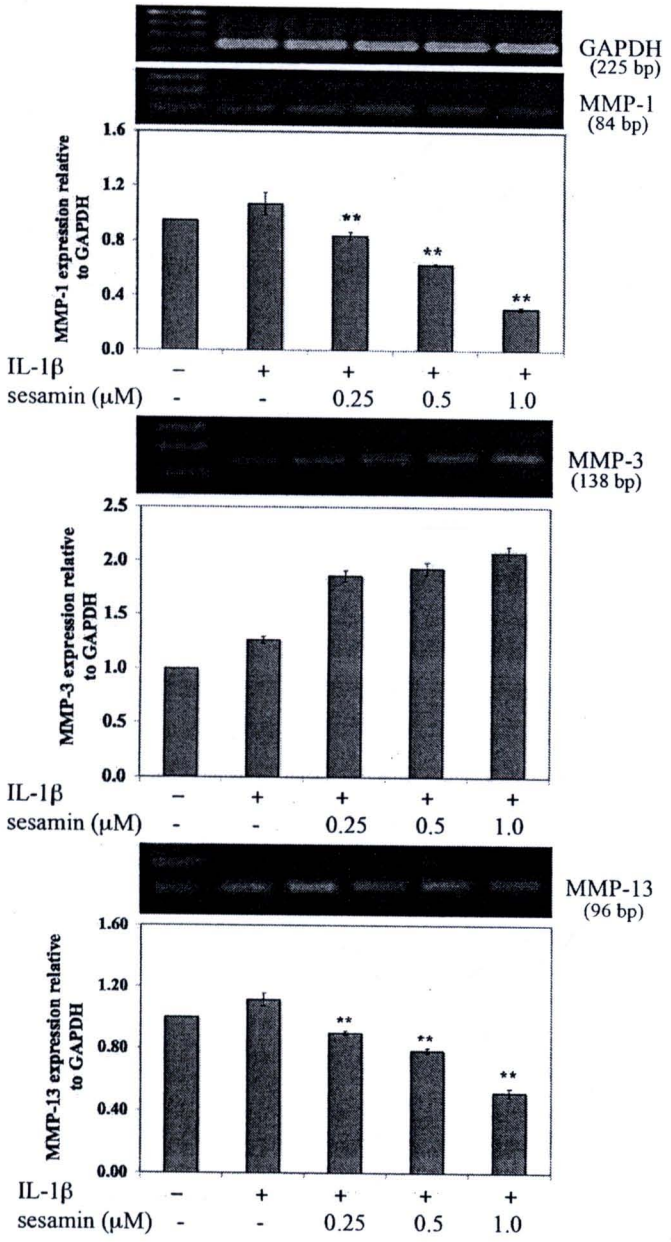

Figure 3.13 Effect of sesamin on mRNA expression of proteinases (MMP-1, -3, -13). Confluent human chondrocytes in 25-cm^3 flasks were cultured with IL-1β (10 ng/ml) in the presence and absence of sesamin for 24 h. Cells were harvested and gene expressions were analyzed.

** denote a value that is significantly different ($p < 0.01$) from the IL-1β control.

For anabolic gene expressions, IL-1β was shown to reduce of the anabolic genes, which are collagen, SOX-9 (the key transcription factor of collagen) and aggrecan core protein (the core protein of proteoglycan that is mainly found in cartilage). It was found that *p*-hydroxycinnamaldehyde and sesamin could reverse the effect of IL-1β on anabolic gene expression (figure 3.14 and 3.15).

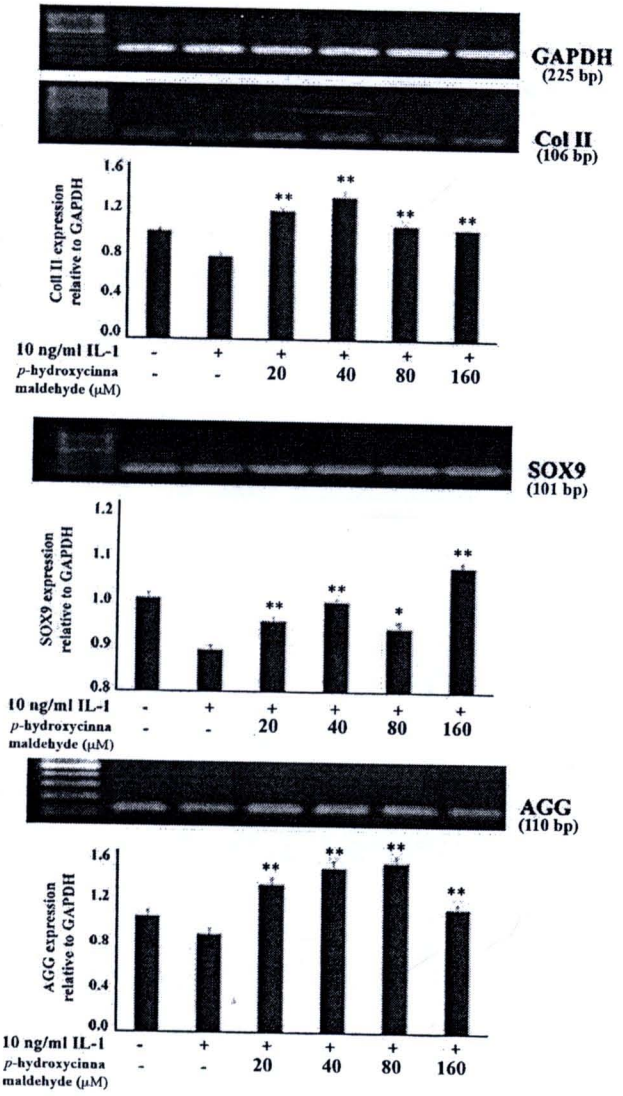

Figure 3.14 Effects of *p*-hydroxycinnamaldehyde on mRNA expression of cartilage genes (AGG, COL2 and SOX9). Confluent human chondrocytes in 25-cm^3 flasks were cultured with IL-1β (10 ng/ml) in the presence and absence of *p*-hydroxycinnamaldehyde for 24 h. Cells were harvested and gene expressions were analyzed. AGG, aggrecan; COL2, collagen type II; SOX9, SRY-type HMG box.

* and ** denote a value that is significantly different ($p < 0.05$ and $p < 0.01$, respectively) from the IL-1β control.

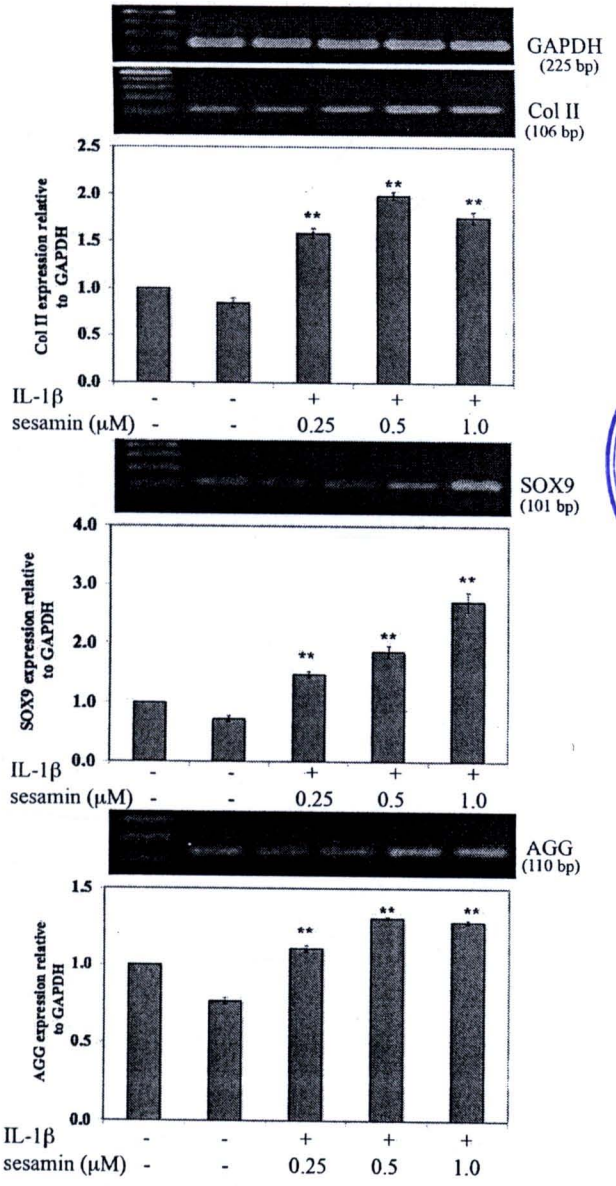

Figure 3.15 Effects of sesamin on mRNA expression of cartilage genes (AGG, COL2 and SOX9). Confluent human chondrocytes in 25-cm^3 flasks were cultured with IL-1β (10 ng/ml) in the presence and absence of sesamin for 24 h. Cells were harvested and gene expressions were analyzed. AGG, aggrecan; COL2, collagen type II; SOX9, SRY-type HMG box. ** denote a value that is significantly different ($p < 0.01$) from the IL-1β control.

As shown in previous results that the effective doses of *p*-hydroxycinnamaldehyde (20-160 μM) and sesamin (0.25-1.00 μM) were different. The effective doses of sesamin was lower than *p*-hydroxycinnamaldehyde. The highest dose of both *p*-hydroxycinnamaldehyde and sesamin could inhibit IL-1β effects more than 80% in almost tested effects. However, the highest dose of sesamin was lower than *p*-hydroxycinnamaldehyde about 160 times. It could be suggested that sesamin had more chondroprotective effect than that of *p*-hydroxycinnamaldehyde. Altogether, sesamin was chosen for further study the condroprotective effects in long-term porcine cartilage explant culture, the molecular mechanisms and its effect in the *in vivo* papain induced OA rat model.

3.4 Investigation of chondroprotective effect of sesamin in long-term porcine cartilage explant culture induced inflammation using IL-1β

3.4.1 The effect of sesamin on proteoglycans (PGs) degradation

To confirm chondroprotective effect of sesamin, long-term porcine cartilage explant was used as a model. Thirty to 35 mg cartilage discs were cultured in 24-well plate and then co-treated with 10 ng/ml IL-1β and 10 μM sesamin for 28 days. Conditioned media were collected and changed at day 4, 7, 14, 21 and 28 of experimental period. The degradation of PGs and collagen in cartilage tissue were investigated by measurement of s-GAG and hydroxyproline release in conditioned media. IL-1β significantly induced the releases of s-GAG at day 4 and 7 around 1.8 and 2 times, respectively when compared with those of the control and these inductions were significantly inhibited by 1.0 μM sesamin (figure 3.16 (A)). The ability of sesamin on inhibition of IL-1β-induced PGs degradation was confirmed by

measurement of uronic acid remaining in cartilage tissue. It was found that uronic acid remaining was decreased by IL-1β, this effect was also inhibited by sesamin (figure 3.16 (B)) indicating that sesamin could inhibit PGs degradation in IL-1β induced cartilage.

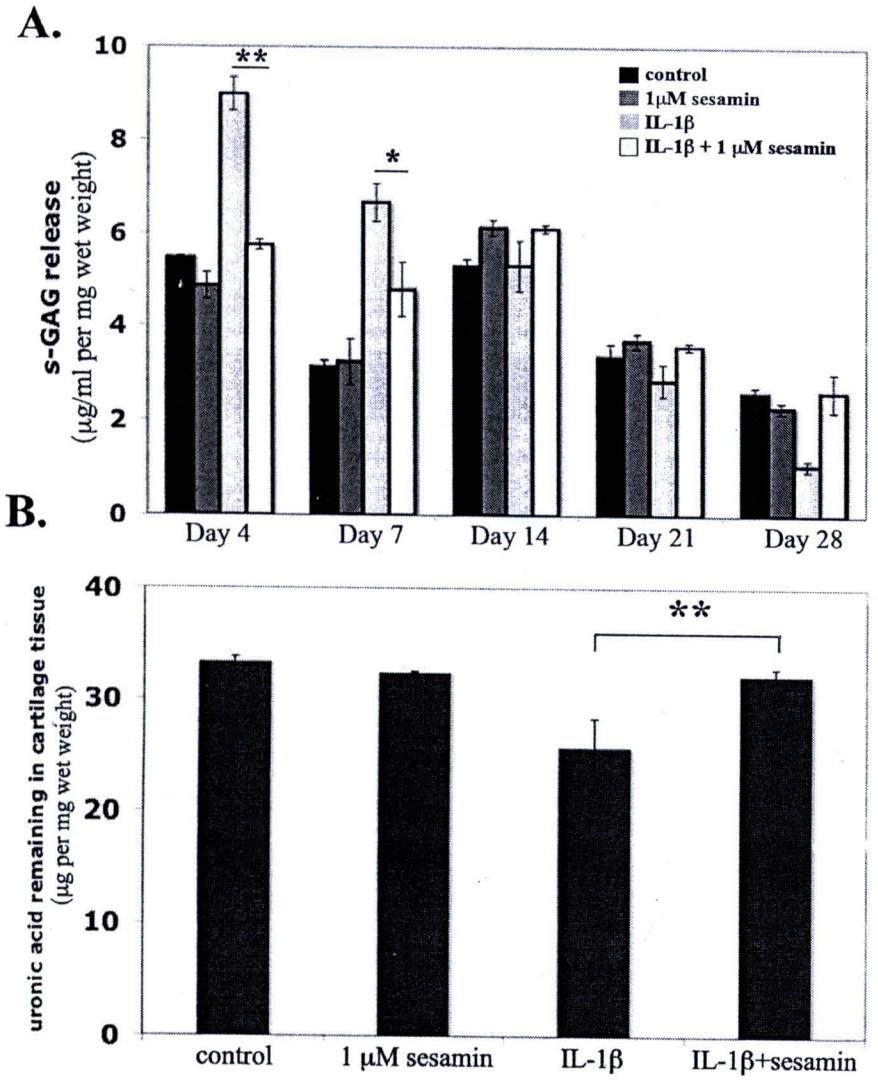

Figure 3.16 Sulfated GAG release (A) and uronic acid remaining (B) in porcine cartilage tissue. Porcine cartilage discs had been cultured with IL-1β and/or 1 μM sesamin for 28 days. The conditioned media were collected and changed at day 4, 7, 14, 21 and 28. The releases of S-GAG were measured in media using dye-binding assay and the remaining of uronic acid were measured in papain-digested cartilage discs.

*, ** denote *p* value in t-test is lower than 0.05 and than 0.01 respectively.

3.4.2 The effect of sesamin on collagen degradation

IL-1β alone could not induce the degradation of collagen as shown in the unchanged release of hydroxyproline (figure 3.17 (A)). There was the report described that oncostatin M (OSM) has been found in inflammatory disease such as arthritis and multiple sclerosis and plays role on arthritis is the capacity to synergize the action of other inflammatory cytokines: IL-1, TNF-a, IL-17 and LPS (246). Thus, the co-treatment of cartilage with IL-1β and OSM was investigated and found that IL-1β/OSM could induce the release of hydroxyproline release at day 21 and 28 (figure 3.18). Sesamin had ability to invert IL-1β/OSM effect on the release of hydroxyproline (figure 3.18 (A)), confirming with the remaining of hydroxyproline in cartilage tissue (figure 3.18 (B)). Altogether, these results revealed that sesamin had chondroprotective effects showing by inhibition of IL-1β- induced PGs degradation and inhibition of IL-1β/OSM- induced collagen degradation.

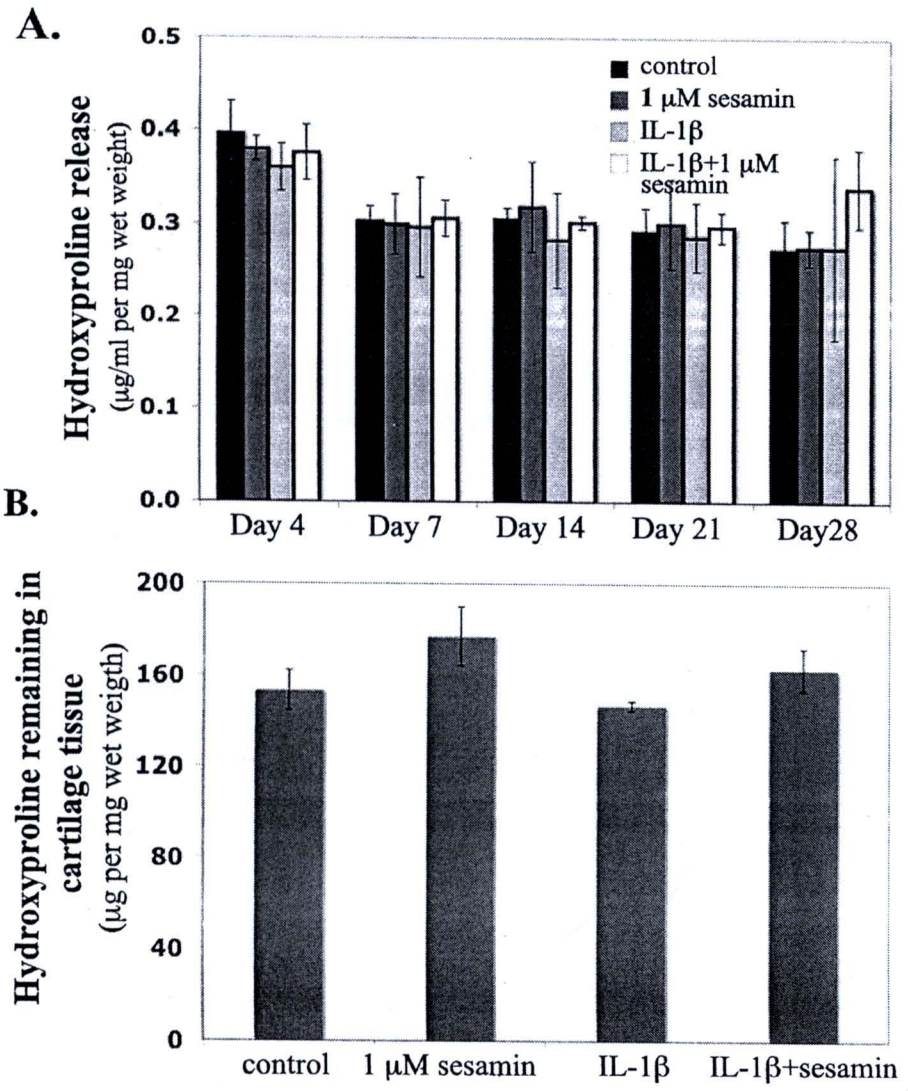

Figure 3.17 The hydroxyproline release (A) and remaining in porcine cartilage explant (B). Porcine cartilage discs had been cultured with IL-1β and/or 1 μM sesamin for 28 days. The conditioned media were collected and changed at day 4, 7, 14, 21 and 28. The release and remaining of hydroxyproline were measured in HCl hydrolyzed-media and cartilage disc respectively as described in experimental procedures.

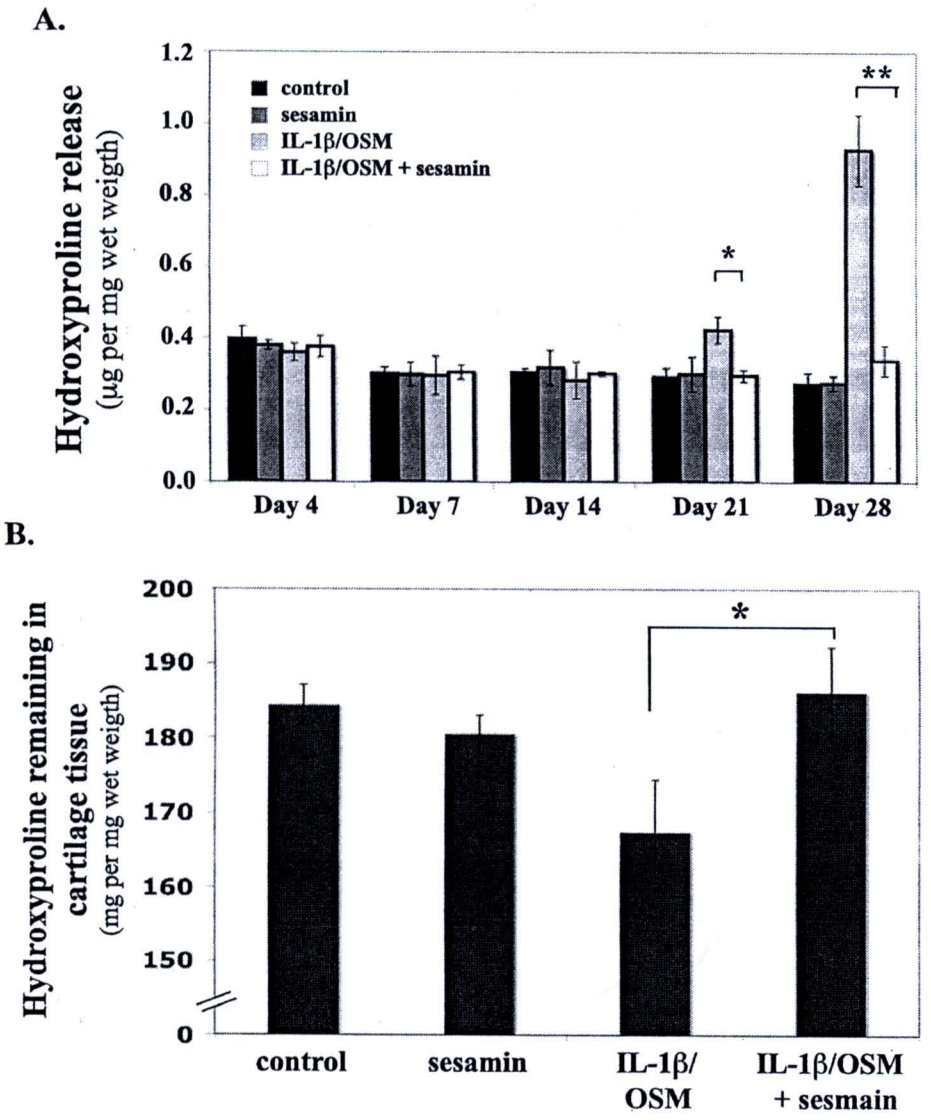

Figure 3.18 The hydroxyproline release (A) and remaining in porcine cartilage explant (B). Porcine cartilage discs had been cultured with IL-1β/OSM and/or 1 μM sesamin for 28 days. The conditioned media were collected and changed at day 4, 7, 14, 21 and 28. The release and remaining of hydroxyproline were measured in HCl hydrolyzed-media and cartilage disc respectively as described in experimental procedures. *, ** denote *p* value in t-test is lower than 0.05 and than 0.01 respectively.

3.5 Investigation molecular mechanism of sesamin chondroprotective effect

3.5.1 The effect of sesamin on ADAMTS activities

As described above that sesamin could inhibit PGs and collagen degradations in cartilage tissue treated with IL-1β. Aggrecan, which is the main PG found in cartilage tissue, is degraded by a number of proteinases including MMPs, ADAMTS species, neutrophil elastase, cathepsin G and B (247). Many MMPs, including MMP-1, -2, -3, -7, -8, -9, -13 and MT1-MMP preferentially cleave the Asn341-Phe342 bond (the MMP site) of aggrecan (248) and ADAMTS-4 (146) and -5 (145) clip the Glu373-Ala374 bond (the aggrecanase site). For collagen, fibrillar type II collagen is the minor collagen found in cartilage. Only MMPs including the classical collagenases (MMP-1, -8 and -13) and MT1-MMP can degrade fibrillar collagen (249).

Thus here we investigated the effect of sesamin on aggrecanase activities and MMP expressions. The activities of aggrecanases were investigated using the reaction of BC-3 antibody, which can react with aggrecanase cleavage site on aggrecan core protein. The conditioned media of cartilage explant (day 4) were used to investigate and it was found that IL-1β could induce the degradation of aggrecan by aggrecanases as indicated with the appearance of BC-3 epitope (figure 3.19). Sesamin could not revert induced-aggrecanase activities (figure 3.19).

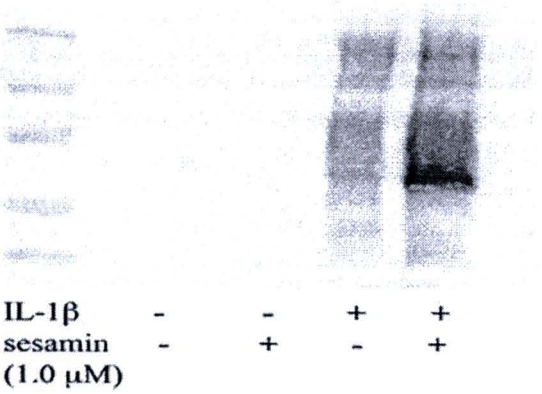

Figure 3.19 Aggrecanase activities, Conditioned media of cartilage explant treated with IL-1β (10 ng/ml) and/or 1 μM sesamin for 4 days were used to inject into SDS-PAGE and transferred to membrane. BC-3 was used to probe the membrane. Alkaline phosphatase conjugated anti-mouse IgG was used as secondary antibody. The reaction was developed using alkaline phosphatase substrate.

3.5.2 The effect of sesamin on MMP-1, MMP-3 and MMP-13 expressions

For effect of sesamin on MMPs, HAC were co-treated with IL-1β and various concentrations of sesamin for 24 h. The mRNA and protein expressions of MMP-1, -3 and -13 were studied. IL-1β significantly induced all MMP-1, -3 and -13 both mRNA and protein levels (figure 3.20 and 3.21). Interestingly, sesamin could revert these effects of IL-1β on all MMP-1, -3 and -13 (figure 3.20 and 3.21). Moreover, in the detail, the activation of all MMP-1, -3 and -13 were increased by IL-1β (indicating in active form). The activations of MMP-1 and -13 might not be influenced by sesamin, but MMP-3 activation significantly disappeared when there was sesamin in condition (figure 3.21). Altogether, sesamin had chondroprotective effect and this might be due to its ability to suppress MMP-1, -3 and -13 expressions in IL-1β induced HAC model.

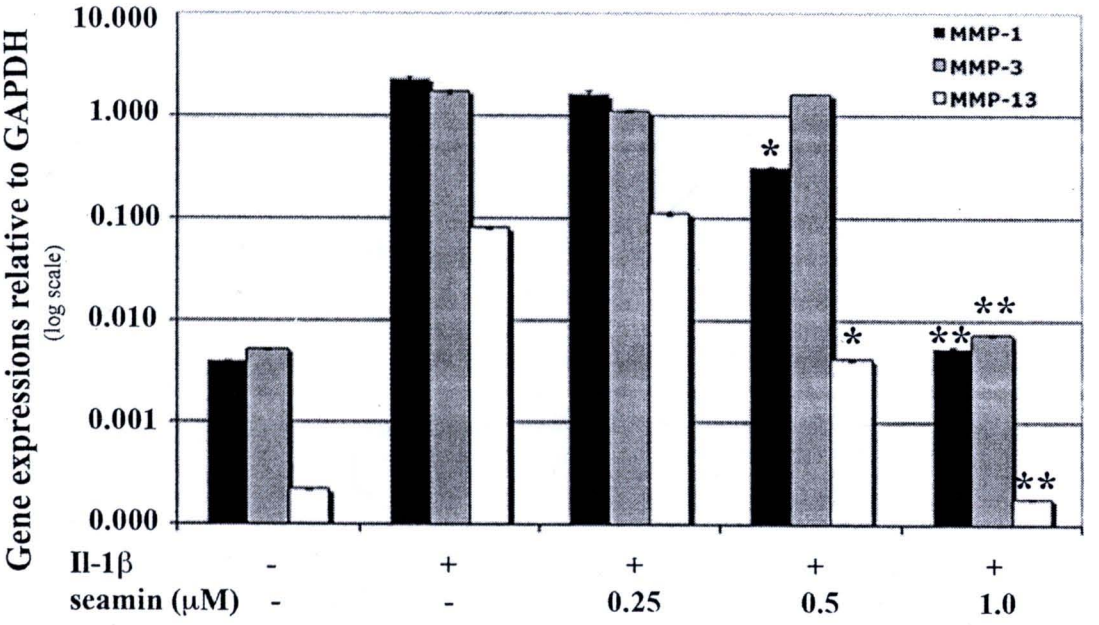

Figure 3.20 MMP-1, -3 and -13 mRNA expressions in HAC treated with IL-1β, Eighty percent confluent of HACs were co-treated with 10 ng/ml IL-1β and various concentrations of sesamin (0.25, 0.5 and 1.0 μM) for 24 h. Cells were harvested and mRNA was isolated. Complementary DNA was generated by reverse transcription PCR. The mRNA of these genes were investigated using real-time PCR with primer as described in experiments and procedures.

*, ** denote *p* value in t-test is lower than 0.05 and than 0.01 respectively.

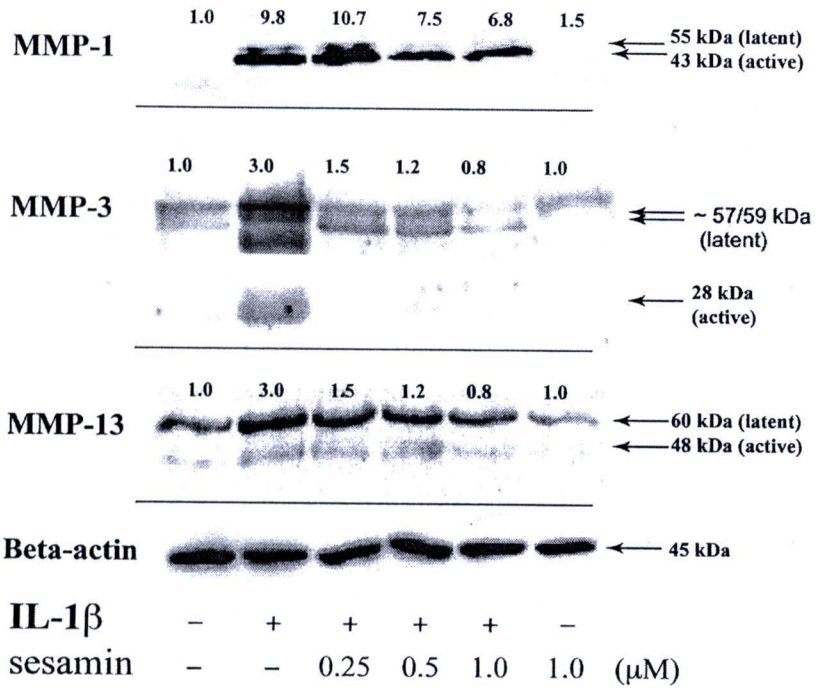

Figure 3.21 MMP-1, -3 and -13 protein expressions in HAC treated with IL-1β, Eighty percent confluent of HACs were co-treated with 10 ng/ml IL-1β and various concentrations of sesamin (0.25, 0.5 and 1.0 μM) for 24 h. Conditioned media were collected and concentrated using centrifugal filter column (10kDa). Filtrated- media were used to injected into SDS-PAGE and transferred to membrane. MMP-1, -3 and -13 protein levels were investigated using specific antibodies as described in experiments and procedures.

3.6 Investigation of sesamin effect on IL-1β signal transduction in HACs

3.6.1 The effect of sesamin on IL-1β induced MAPK signaling pathway in HAC

The best characterized cascade of IL-1β signaling involves NFκB, the three other activate the terminal MAPKs, JNK, p38, and ERK, that transmit signals from extracellular stimuli to activate transcription factors (250, 251).

Prior to investigate the effect of sesamin on IL-1β-induced MAPK signaling pathway in HACs, the activation of p38, ERK and JNK in HACs by IL-1β was studied. Figure 3.22 showed that the activation of p38 and JNK was significantly increased when HACs were treated with IL-1β for 15 and 30 minutes. These activations were auto-dephospherylated at 45 and 60 minutes of treatment. In contrast, for ERK, its activation was slightly increased at 15 minutes of treatment and de-activation at 30 minutes of treatment. Thus, in the next experiment HACs would be treated with IL-1β for 15 minutes for investigation of the effect of sesamin on these activations.

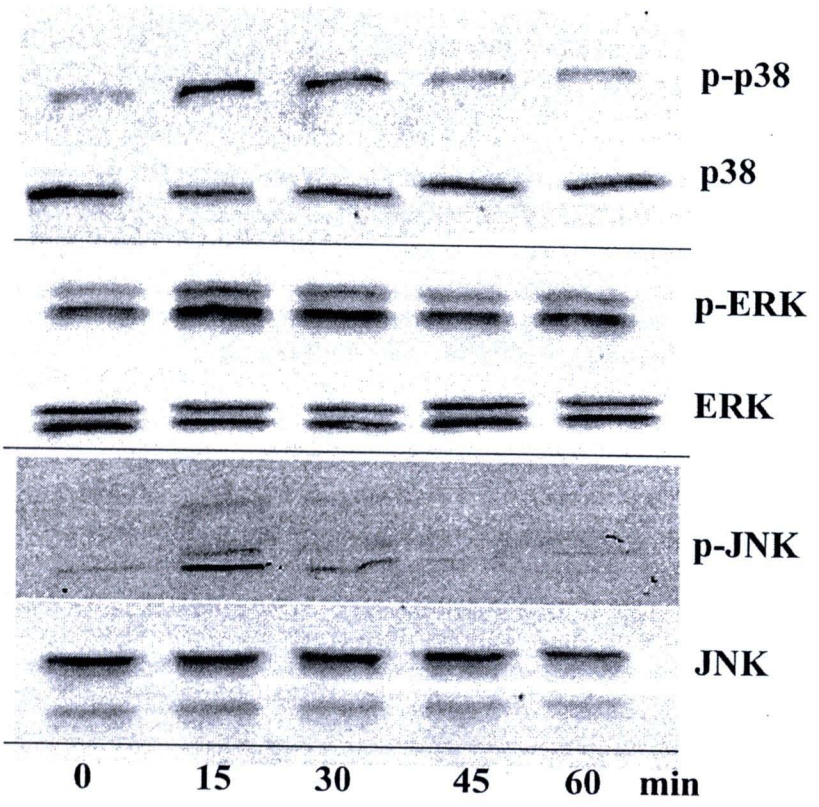

Figure 3.22 The effect of IL-1β on the phosphorylations of MAPK protein families. HACs were treated with 10 ng/ml IL-1β for 15, 30, 45 and 60 min for investigated the activations of MAPK pathways. Cell lysates were extracted using lysis buffer and were analyzed using western blot analysis as described in Materials and Methods.

We studied whether sesamin could inhibit the activations of MAPK family. HACs were co-treated with 10 ng/ml IL-1β and sesamin for 15 minutes. The phosphorylation of p38, ERK1/2 and JNK were studied using immunoblotting. For MAPK pathway, IL-1β significantly induced p38 and JNK phophorylation, ERK1/2 normally was phophorylated and the phophorylated forms of those molecules were increased when IL-1β was presented. Sesamin inhibited IL-1β signals through p38 and JNK but did not trough ERK1/2 (figure 3.23).

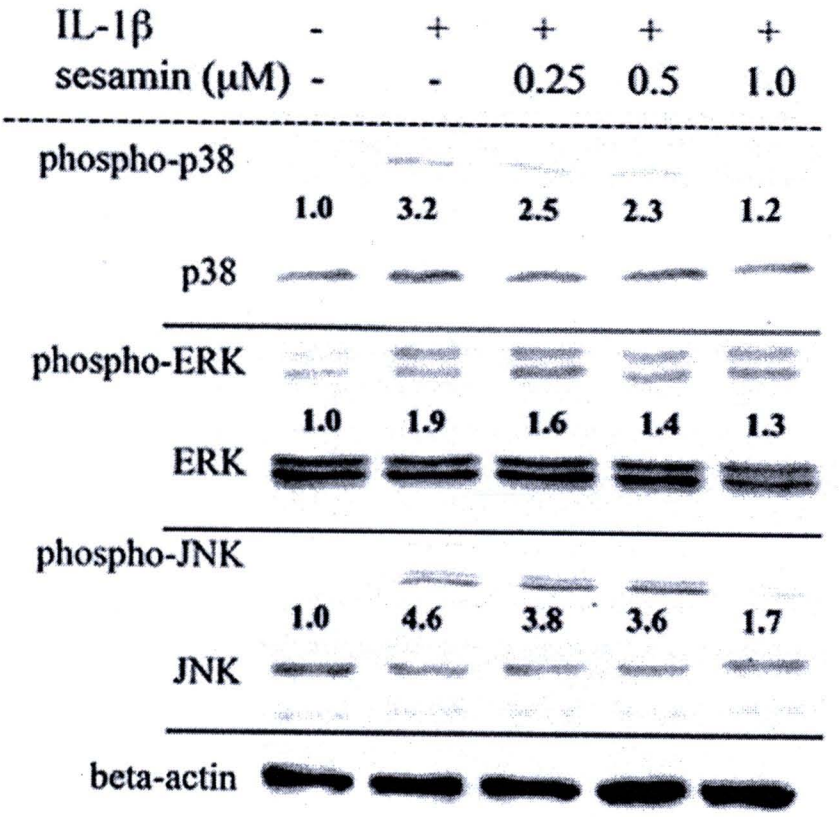

Figure 3.23 The effects of sesamin on the phosphorylations of MAPK protein families. HACs were co-treated with 10 ng/ml IL-1β and sesamin for 15 min for investigating the activations of MAPK pathways. Cell lysates were extracted using lysis buffer and were analyzed using western blot analysis as described in experiments and procedures. The density of each sample was measured using Scion Image program and the values below the bands showed their densities after normalized with their total forms.

3.6.2 The effect of sesamin IL-1β induced NF-κB transcription factor in HAC

As same as MAPK pathway, prior to investigate effect of sesamin on IL-1β induces NFκB transcription factor, the activation of this transcription factor by IL-1β was studied. The activation of NFκB occurred due to the activation of IKKα/β. Phosphorylated IKKα/β could activate IκBα by phosphorylation. IκBα normally associated with NFκB transcription factor resulting to inactivation of transcription factor. The phosphorylation of IκBα leaded to dissociated from NFκB transcription factor resulting in the phosphorylation of p65 subunit of NFκB transcription factor and activation of this transcription factor. As shown in figure 3.24, when HACs were treated with IL-1β for 5-10 minutes the activation of IKKα/β, IκBα and p65 subunit of NFκB were occurred and these activation were auto-deactivated when HACs were treated longer than 20 minutes. Thus, in the next experiment HACs would be treated with IL-1β for 10 minutes to investigate the effect of sesamin on IL-1β induced NFκB activation.

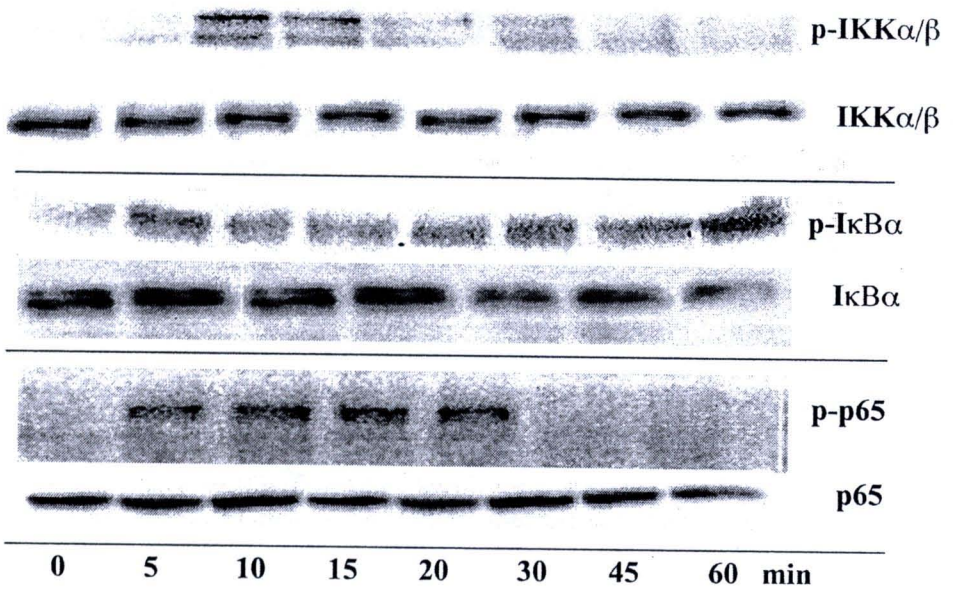

Figure 3.24 The effect of IL-1β on the phosphorylations of IKKα/β, IκBα and p65 subunit of NFκB transcription factor. HACs were treated with 10 ng/ml IL-1β for 5, 10, 15, 20, 30, 45 and 60 min for investigated the activations of NFκB transcription factor. Cells were extracted using lysis buffer. Cell extracts were analyzed using western blot analysis as described in Materials and Methods.

As described above the activation of NFκB by IL-1β occurs by the phophorylaton of IKKα/β leading to the phosphorylation of IκBα, resulting of free phosphorylated NFκB p65. The IL-1β-induced-IKKβ phophorylation was inhibited by sesamin in the dose dependent manner leading to the reduction of IκBα phophorylation and the reduction of NFκB transcription factor activation as shown in the reduction of the phosphorylation of p65 subunit of NFκB (figure 3.25).

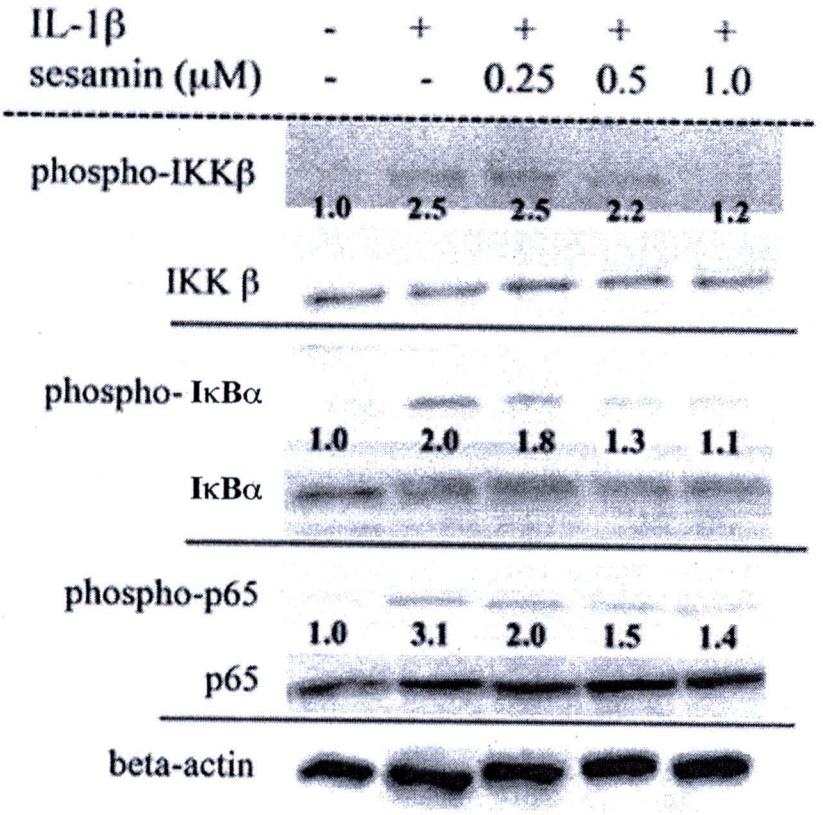

Figure 3.25 The effects of sesamin on the phosphorylations of IKKα/β, IκBα and p65 subunit of NFκB transcription factor. HACs were co-treated with 10 ng/ml IL-1β and sesamin for 10 min for investigating the activations of NFκB transcription factor. Cells were extracted using lysis buffer. Cell extracts were analyzed using western blot analysis as described in experiments and procedures. The density of each sample was measured using Scion Image program and the values below the bands showed their densities after normalized with their total forms.

3.7 Investigation of sesamin effect on osteoarthritis pathological progression in papain-induced osteoarthritis (OA) rat model

3.7.1 The effect of sesamin on the cartilage and chondrocyte morphology in papain-induced osteoarthritis (OA) rat model

Rats were induced OA condition by using papain injection into the knee (234). Using H&E staining, cartilage in control rats had a normal thickness with well organize zones and columns of chondrocytes (figure 3.26 (A)). The 10 μM sesamin group exhibited similar results (figure 3.26 (B)). Cartilage in OA group showed significant morphologic changes including thinning articular cartilage and disorganized chondrocytes (figure 3.26 (C)). OA group treated with 1 and 10 μM sesamin showed thicker cartilage and better organization of chondrocytes when compared with OA rats (figure 3.26 (D, E)).

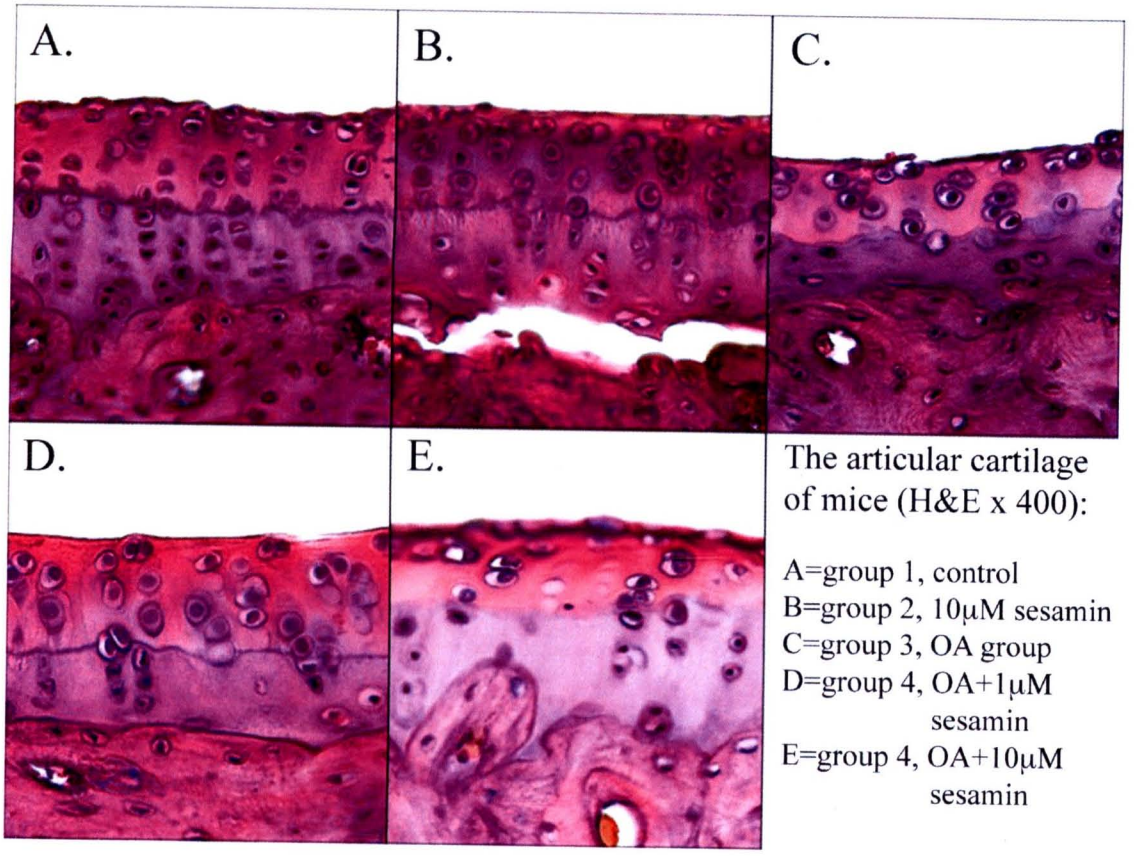

Figure 3.26 H&E staining in cartilage of normal rats (A), mornal rats+10 μM sesamin (B), papain-induced OA rats (C), OA rats+1 μM sesamin (D) and OA rats+10 μM sesamin (E). Rats were induced OA using papain injection into knee. After induction period 1 or 10 μM sesamin was injected into the knees of rats every 5 days for 25 days. Rats were sacrificed at the end of experiment and sections of knees' rats were performed. Some sections were stained with H&E to investigate cell morphology.

3.7.2 The effect of sesamin on the degradation of extracellular matrix (ECM) molecules in papain-induced osteoarthritic (OA) rat model

The PGs in cartilage tissue were observed by safranin O staining. The 10 μM sesamin-treated group showed the strongest intensity of safranin O staining (figure 3.27 (A)). The lowest intensity of safranin O staining was found in the OA group (figure 3.27 (C)). Treatment of OA group with 1 and 10 μM sesamin showed stronger intensity of safranin O staining in the dose dependent when compared with that of the OA group (figure 3.27 (D-E)).

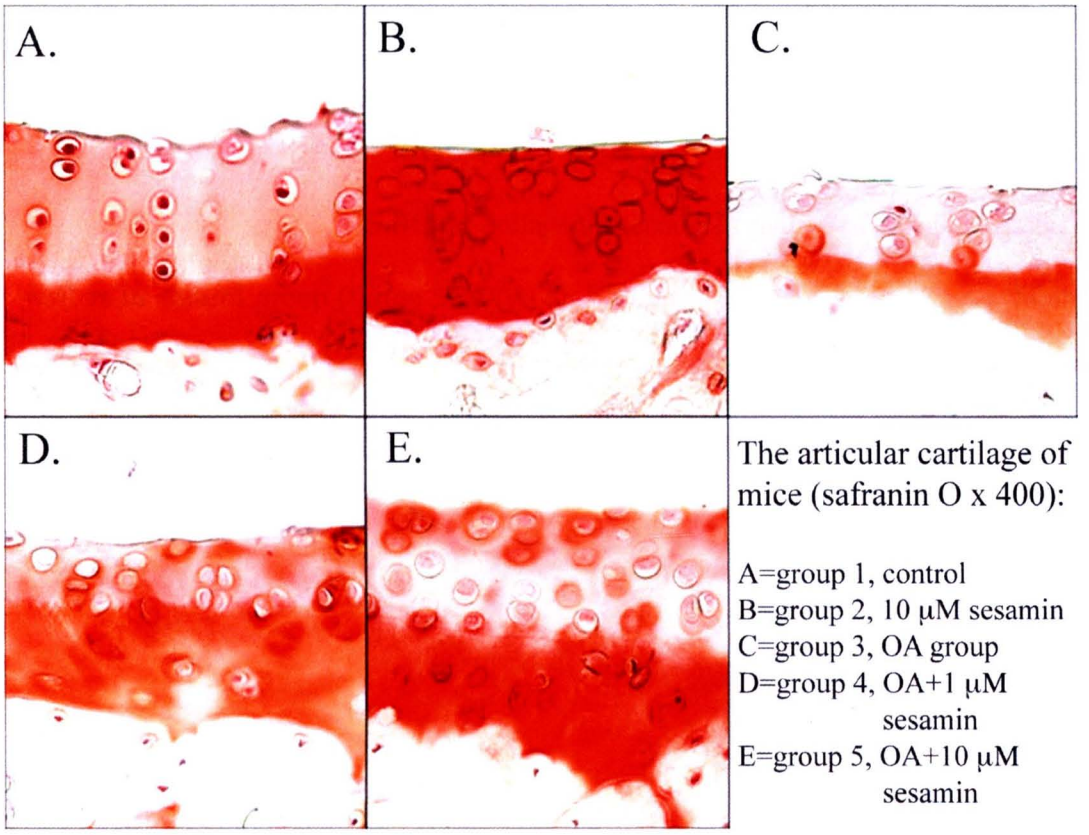

Figure 3.27 Safranin O staining in cartilage of normal rats (A), mornal rats+10 μM sesamin (B), papain-induced OA rats (C), OA rats+1 μM sesamin (D) and OA rats+10 μM sesamin (E). Rats were induced OA condition by using papain injection into knee. After induction period 1 or 10 μM sesamin was injected into the knees of rats every 5 days for 25 days. Rats were sacrificed at the end of experiment and sections of knees' rats were performed. Some sections were stained with safranin O to investigate the content of PGs in cartilage matrix.

Another major molecule found in cartilage was investigated, the immunohistochemistry for type II collagen was performed. OA group showed weak and focal collagen intensity (figure 3.28 (C)). OA rats treated with 1 and 10 μM sesamin showed strong and diffuse collagen intensity in matrix of cartilage tissue as well as in control group (figure 3.28 (A, D, E)). The 10 μM sesamin group exhibited stronger collagen intensity than that in control group (figure 3.28 (B)).

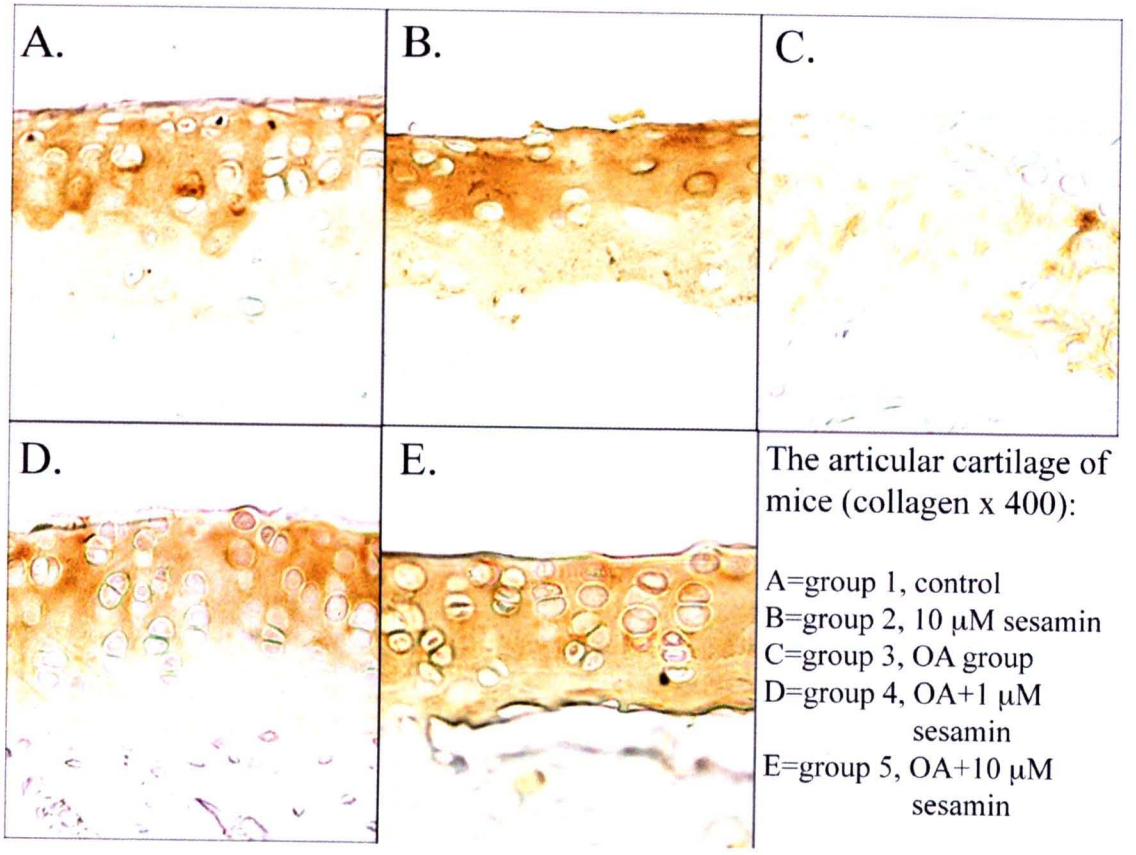

Figure 3.28 Type II collagen immunohistochemical staining in cartilage of normal rats (A), mornal rats+10 μM sesamin (B), papain-induced OA rats (C), OA rats+1 μM sesamin (D) and OA rats+10 μM sesamin (E). Rats were induced OA condition by using papain injection into knee. After induction period 1 or 10 μM sesamin was injected into the knees of rats every 5 days for 25 days. Rats were sacrificed at the end of experiment and sections of knees' rats were performed. Some sections were immunohistochemical stained with anti-type II collagen antibody to investigate the content of type II collagen in cartilage matrix.

Taken together, the results revealed that sesamin could inhibit the pathological progression of papain-induced OA rats, moreover, sesamin alone might had effect on the increases of PGs and collagen in cartilage matrix of non-treated rats.

3.8 Investigation of the chondroprotective effect of the combination between sesamin and glucosamin-sulfate

3.8.1 Comparison of glucose derivatives effects on cartilage degradation

As described in introduction part that nowadays glucosamine is used to treat OA patients. Glucosamine (GlcN) used for OA treatment is mostly GlcN derivatives, such as glucosamine-hydrochloride (GlcN-HCl) and glucosamine-sulfate (GlcN-S). There are some reports that compare the effects of these derivatives. It was found that GlcN-S is a stronger inhibitor of gene expression than GlcN-HCl (252). However, there has to date been no comparison of the chondroprotective effects of GlcN derivatives. In this study, we compared the chondroprotective effects of GlcN-HCl, GlcN-S, glucose (Glc) and glucuronic acid (GlcA) in porcine cartilage explants and human articular chondrocytes (HAC) that had been induced degradation by IL-1β. Since the metabolic imbalance in OA includes both an increase in cartilage degradation and insufficient reparative or anabolic response (253), the effects of these glucose derivatives, on both catabolic and anabolic gene expression, were studied and compared in HAC treated with IL-1β. The one of GlcN derivatives, which showed the highest chondroprotective effect would be chosen to study the combination effect with sesamin.

3.8.1.1 Chondroprotective effects of Glc, GlcN-S, GlcA and GlcN-HCl in porcine cartilage explants

Porcine cartilage explants were induced to degrade by using 10 ng/ml IL-1β and the chondroprotective effects of all four chemicals were studied by co-treatment with IL-1β and each chemical (20, 40, 80 mM) for 3 days. Conditioned media were collected and analyzed. Extracellular matrix (ECM) molecules were used as the indicators. Since, IL-1β induces the degradation of ECM molecules in cartilage discs, and degraded ECM molecules are released into the media while undegraded molecules remain in the cartilage tissue.

The release of HA and s-GAG, which are ECM molecules, from cartilage tissue into media were analyzed by ELISA and dye-binding assays, respectively. Gelatin zymography was used to measure MMP-2 activity. The remnant ECM molecules were measured by digestion of conditioned cartilage with papain, followed by measuring the remaining uronic acid.

IL-1β induced the release of HA and s-GAG from cartilage into media (figure 3.29 (A, B)). GlcN-S, GlcN-HCl and GlcA decreased HA release. Among these three chemicals, GlcN-S exhibited the highest inhibitory effect. However, HA released to the media was not reduced by Glc (figure 3.29 (A)). For s-GAG releases, GlcN-S and GlcN-HCl had the ability to reduce s-GAG release while Glc and GlcA did not. GlcN-S also had the highest inhibitory effect on s-GAG release. Moreover, IL-1β induced the activity of MMP-2 (figure 3.30). This induced activity was decreased by GlcA, GlcN-HCl and GlcN-S, but not by Glc. Similarly to HA and s-GAG, GlcN-S possessed the highest inhibitory effect on MMP-2 activity.

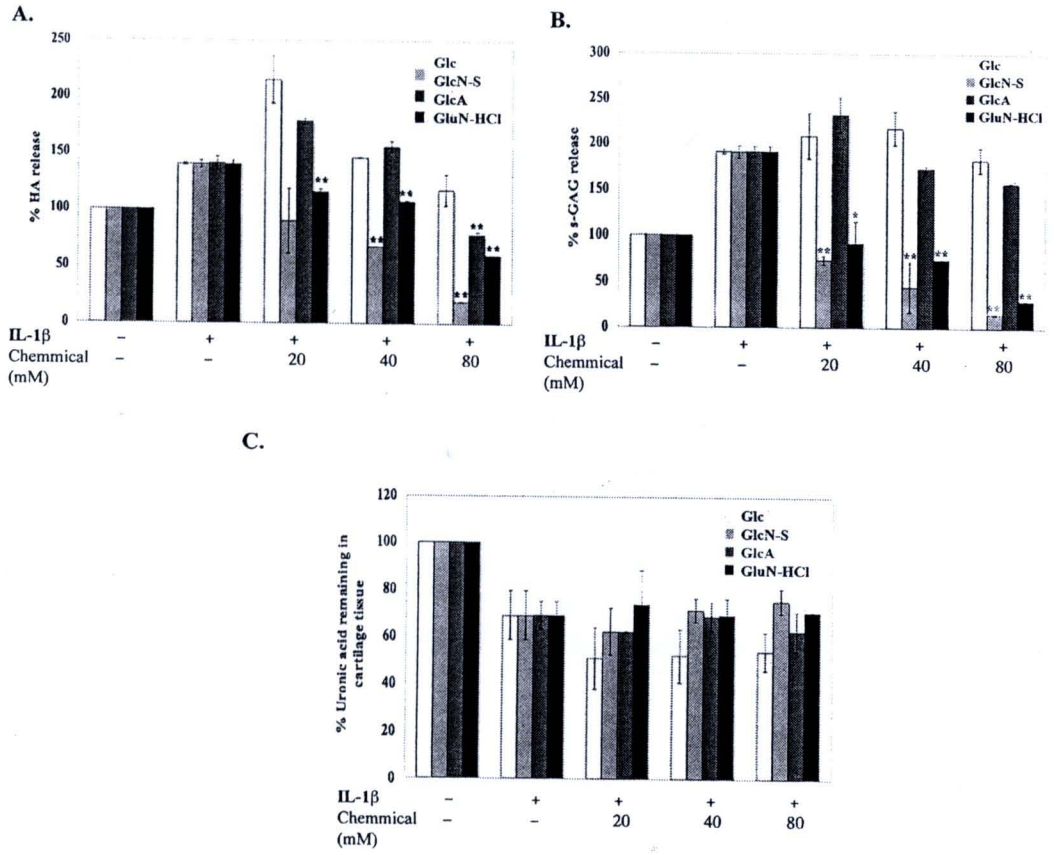

Figure 3.29 The effects of Glc, GlcN-S, GlcA and GlcN-HCl: release of HA (A), s-GAG (B), from porcine cartilage tissues to the media, the uronic acid remaining in the cartilage tissue (C). Porcine cartilage explants were cultured with IL-1β (10 ng/ml) in absence and presence of each chemical (at varying concentrations of 20, 40, 80 mM) for 3 days. In the media, the s-GAG release was measured by using a dye-binding assay, and HA release was measured by ELISA. Cartilage discs were digested with papain and then the uronic acid content was measured.

*, ** Denotes: a value that is significantly different ($p<0.05$ and $p<0.01$, respectively) from the IL-1β control.

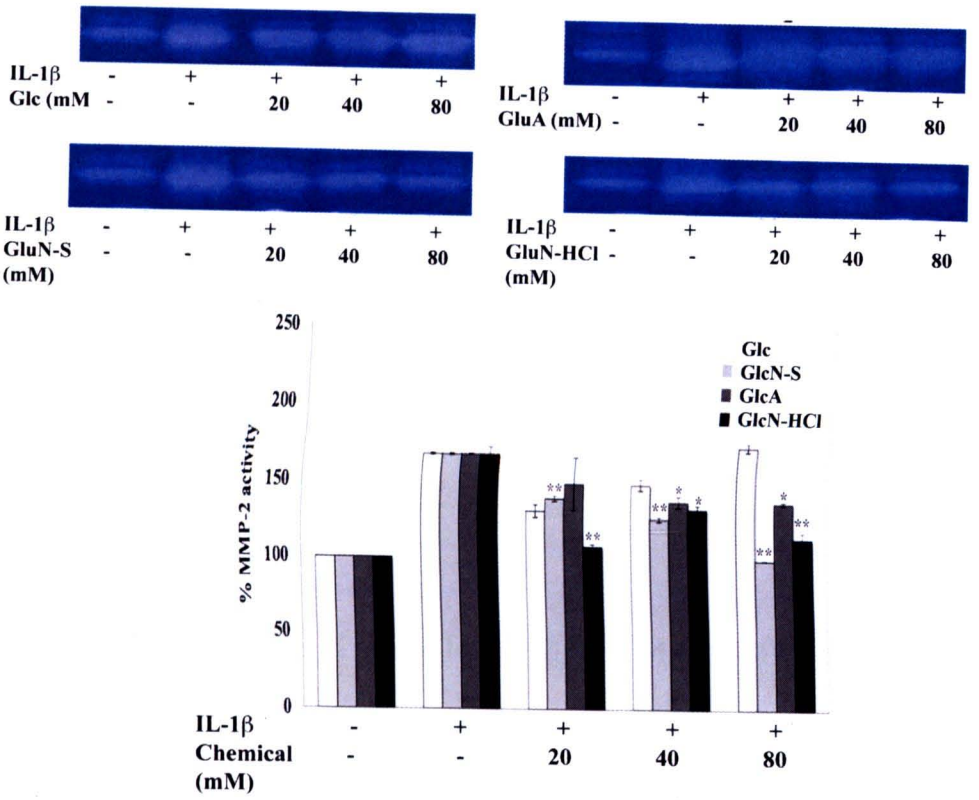

Figure 3.30 Effects of Glc, GlcN-S, GlcA and GlcN-HCl on the production of MMP-2. Porcine cartilage explants were cultured with IL-1β (10 ng/ml) in the absence and presence of each chemical (at varying concentrations of 20, 40, 80 mM) for 3 days. Media were collected and then analyzed by gelatin zymography as described in the Materials and Methods. Three experiments were carried out independently and they were reproducible. *, ** Denotes a value that is significantly different ($p<0.05$ and $p<0.01$, respectively) from the IL-1β control.

For the remaining uronic acid in cartilage discs, when cartilage discs were treated with IL-1β, the remaining uronic acid content was lower than for that of the control group (figure 2.29 (C)). It was found that all four chemicals could not significantly reverse this effect of IL-1β, but GlcN-S and GlcN-HCl trended to inhibit uronic acid loss from cartilage in the highest dose (80 mM). Altogether, these results suggest that GlcN-S had the highest chondroprotective effect among all four chemicals used in our porcine cartilage explant model. We continued by analyzing the chondroprotective effects of all four chemicals in human chondrocytes.

3.8.1.2 Cytotoxic effects of Glc, GlcN-S, GlcA and GlcN-HCl in HAC

The cytotoxic effects of 5, 10 and 20 mM of all four chemicals were initially studied in the HAC model. As shown in figure 3.31, none of the three concentrations of the four chemicals had cytotoxic effects on HAC. Thus, these three concentrations could be used for studying the chondroprotective effects of the chemicals in subsequent experiments.

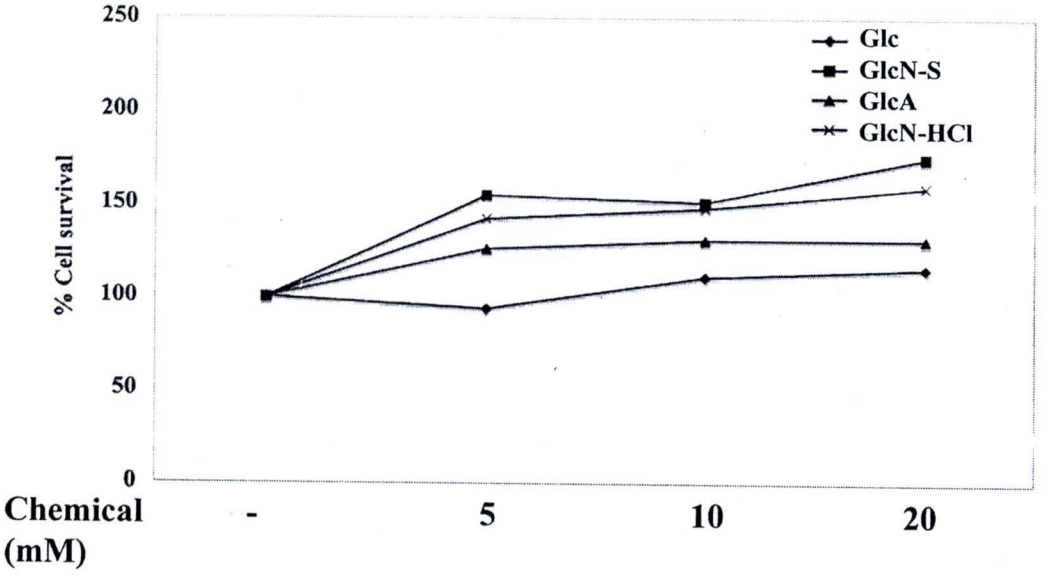

Figure 3.31 The cytotoxic effects of Glc, GlcN-S, GlcA and GlcN-HCl. The cytotoxic effect of all reagents at concentrations 5, 10 and 20 mM in human articular chondrocytes were studied by the MTT assay.

3.8.1.3 Effects of Glc, GlcN-S, GlcA and GlcN-HCl on HA release and MMP-2 activity in IL-1β-treated-HAC

HACs were co-treated with 10 ng/ml IL-1β and 5, 10 and 20 mM of each chemical for 24 hours. The conditioned media were collected and analyzed for HA and MMP-2 activity.

IL-1β was able to induce the release of HA and MMP-2 activity into the media (figure 3.32). The induced HA release was inhibited by GlcN-S, Glc and GlcN-HCl but was not inhibited by GlcA. GlcN-S showed the highest inhibitory effect, followed by Glc and GlcN-HCl (figure 3.32 (A)). Glc and GlcA did not decrease induced MMP-2 activity, whereas both GlcN-S and GlcN-HCl did so. Among the four chemicals studied, GlcN-S had the highest inhibitory effect (figure 3.32 (B)). In the human chondrocyte model, GlcN-S also had the highest chondroprotective effect.

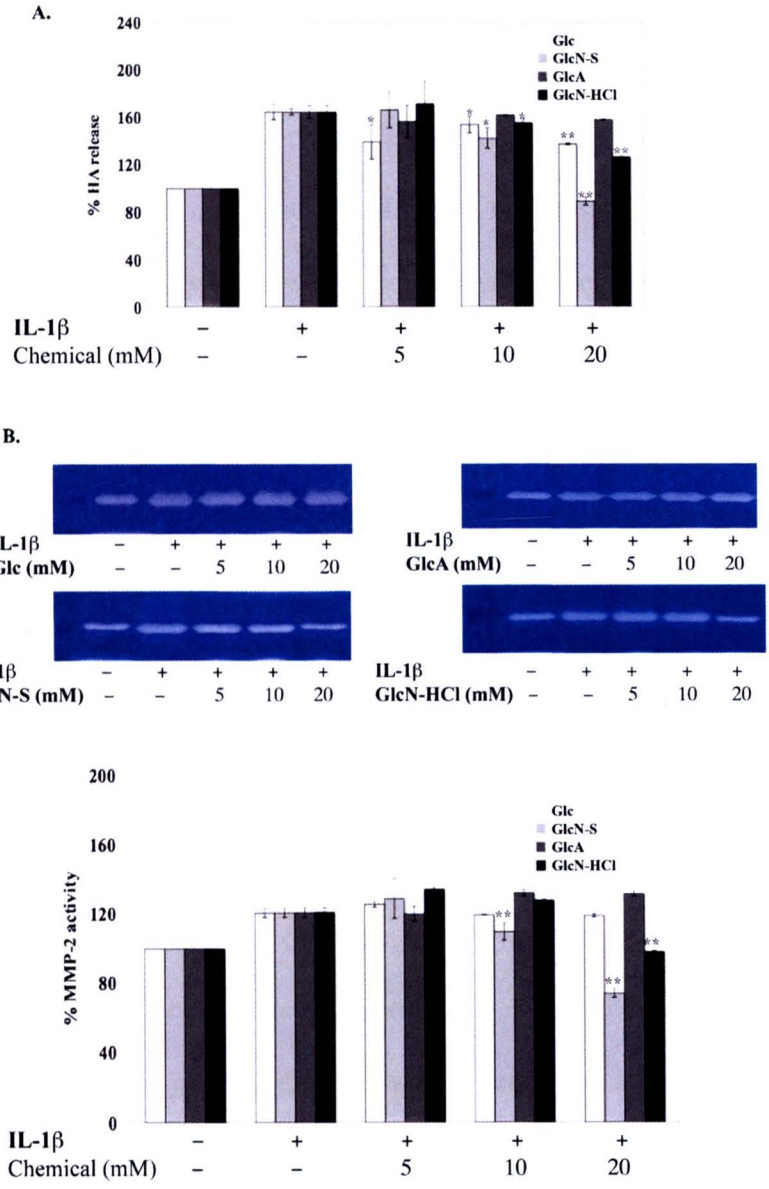

Figure 3.32 Effects of Glc, GlcN-S, GlcA and GlcN-HCl on the release of HA (A), s-GAG (B) and MMP-2 (C) from chondrocytes. Chondrocytes were co-treated with 10 ng/ml IL-1β and various concentrations of each chemical (5, 10, 20 mM) for 24 hours. The conditioned media were analyzed for HA, s-GAG and MMP-2 activity as described in Materials and Methods. *, ** Denotes a value that is significantly different ($p<0.05$ and $p<0.01$, respectively) from the IL-1β control.

3.8.1.4 Effects of Glc, GlcN-S, GlcA and GlcN-HCl on catabolic gene expression in HAC

To study gene expression, HACs were co-treated with 10 ng/ml IL-1β and 5, 10 and 20 mM of each chemical for 24 hours. Cell lysates were harvested and then extracted for mRNA, which was used to synthesize cDNA. The RT-PCR was performed using the primers as described in Materials and Methods.

It has been well documented that the expression levels of many genes change in cultured chondrocytes as compared to that of intact cartilage and, moreover, between the cultured passage (254-257). To avoid the variations of the expression levels between passages, the genes showed negligible change between passage cultures were chosen for investigation. It was found that the expression of MMP-3, MMP-13, aggrecan core protein (AGG) and SOX-9 (a transcriptional factor for type II collagen) were not significantly changed between fresh isolated chondrocytes (passage 2) and used passage (passage 4) (258). Thus, MMP-3 and MMP-13 were chosen as catabolic genes, while AGG and SOX-9 were chosen as anabolic genes.

Regarding the expression of catabolic genes, we studied MMP-3 and MMP-13. Both MMP-3 and -13 were induced by IL-1β (figure 3.33). The induced MMP-3 expression was inhibited by GlcN-HCl and GlcN-S, while GlcN-HCl showing the highest inhibitory effect. Glc and GlcA had no inhibitory effect on expression of either gene. On the contrary, Glc and GlcA seemed to further induce MMP-3 expression. For MMP-13, GlcN-HCl and GlcN-S could inhibit the induction of MMP-13 by IL-1β, and GlcN-S showed the highest inhibitory effect. Glc had no effect on induced MMP-13 expression, but GlcA increased MMP-13 expression.

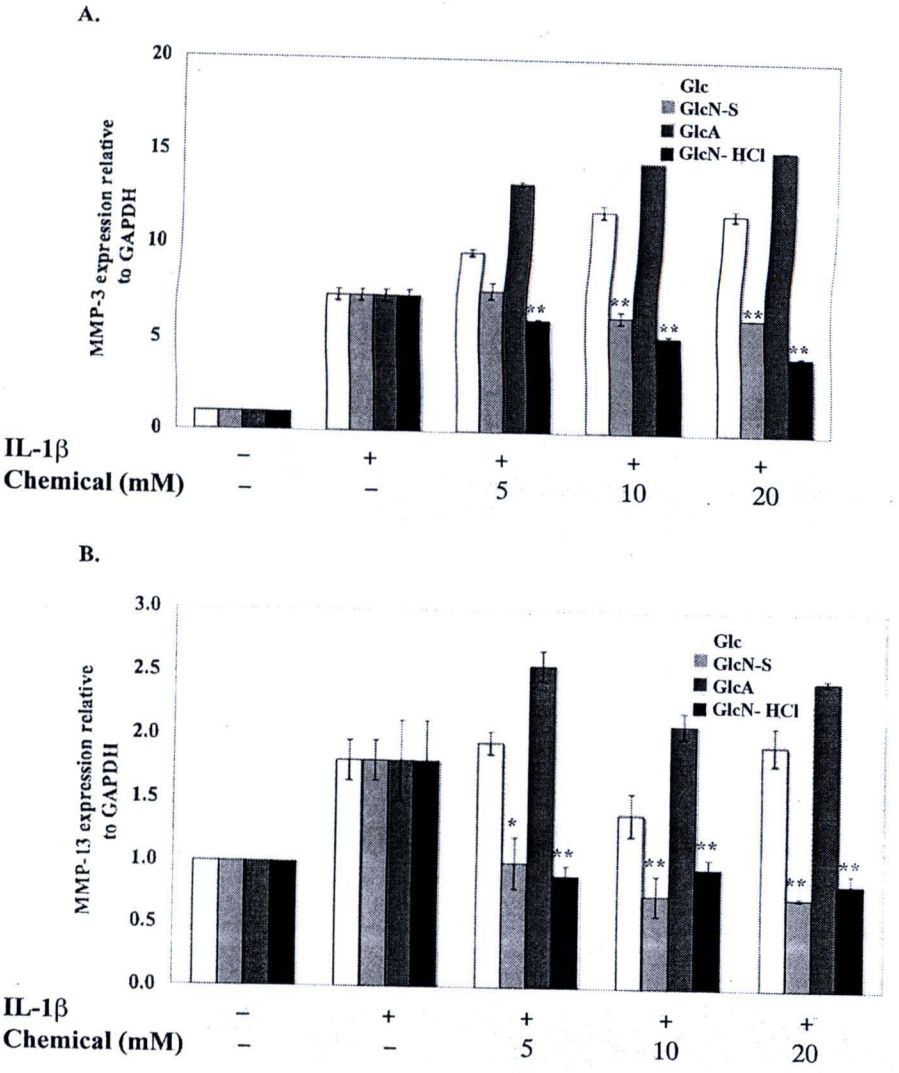

Figure 3.33 Effect of Glc, GlcN-S, GlcA and GlcN-HCl on the mRNA expression of proteinases [MMP-3 (A), -13 (B)]. Confluent human chondrocytes in 25-cm^3 flasks were cultured with IL-1β (10 ng/ml) in the presence and absence of each chemical for 24 hours. Cells were harvested and gene expression was analyzed.

*, ** Denotes a value that is significantly different ($p<0.05$ and $p<0.01$, respectively) from the IL-1β control.

3.8.1.5 Effects of Glc, GlcN-S, GlcA and GlcN-HCl on anabolic gene expressions in HAC

Regarding to the effects of the compounds on anabolic genes, we analyzed the expression of AGG and SOX9. There was no significant difference in expression of either AGG or SOX9 genes when treated with IL-1β (figure 3.34). Glucose and GlcA induced AGG gene expression, while GlcN-HCl and GlcN-S showed reduced effects. The GlcN-HCl group had the highest effect. SOX9 expression was not changed when treated with Glc or GlcN-S, but was increased by GlcA and was decreased by GlcN-HCl.

This study shows that glucosamine derivatives can alter anabolic and catabolic processes in HACs induced by IL-1β. GlcN-S and GluN-HCl decreased induced MMP-3 and -13 expressions, while Glc and GlcA increased reduced-AGG and SOX9 expression. The chondroprotective study using porcine cartilage explant showed that GlcN-S had the strongest effect. Thus, in the next experiment, GlcN-S was chosen to study the combinatory effect with sesamin in porcine cartilage explant to answer that whether sesamin could show synergistic effect with GlcN-S.

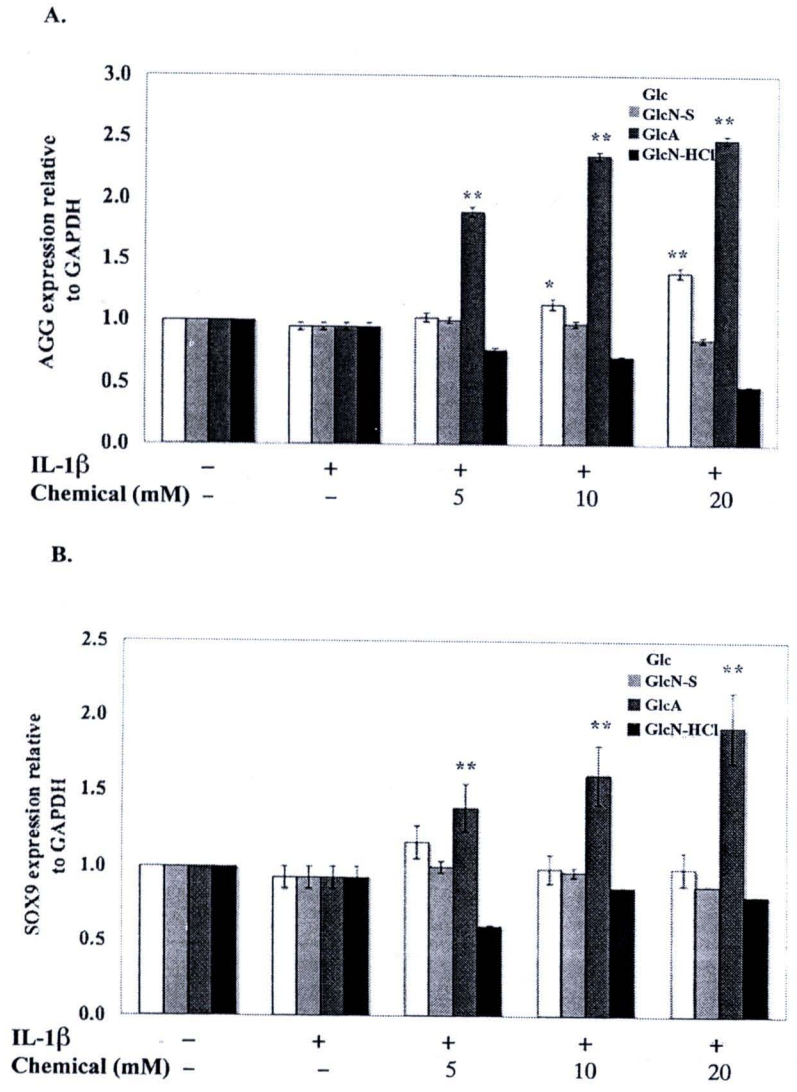

Figure 3.34 Effects of Glc, GlcN-S, GlcA and GlcN-HCl on the mRNA expression of cartilage genes [AGG (A), SOX9 (B)]. Confluent human chondrocytes in 25-cm^3 flasks were cultured with IL-1β (10 ng/ml) in the presence and absence of each chemical for 24 hours. Cells were harvested and mRNA was isolated, then gene expression was analyzed. AGG (aggrecan), SOX9 (SRY-type HMG box).

*, ** Denotes a value that is significantly different ($p<0.05$ and $p<0.01$, respectively) from the IL-1β control.

3.8.2 The additive chondroprotective effect of sesamin with GlcN-S on porcine cartilage explant

To study whether sesamin had additive effect with GlcN-S, porcine cartilage explant was performed. The effective doses of GlcN-S were 5-20 mM while those of sesamin were 0.25-1.0 μM. These dose range of both chemicals were used for cartilage explant treatment. Moreover, to investigate additive effect, those 3 doses were used to co-treat cartilage explant. Other 3 lower doses of both chemicals were also used to study the additive effect.

As shown in figure 3.35 and table 3.1, 80·mM GlcN-S could inhibit HA release around 43% while 1.0 μM sesamin could inhibit about 56%. When IL-1β induced cartilage explants were co-treated with 40 mM GlcN-S and 0.5 μM sesamin, the release of HA was inhibited around 77%. Moreover, when those cartilage explants were treated with 80 mM GlcN-S and 1.0 μM sesamin, the release of HA was reduced around 100% when compared with IL-1β treated alone. These results suggest that sesamin might have additive effect with GlcN-S on inhibition of HA (nonsulfated-proteoglycan) degradation.

For s-GAG release, which indicates the degradation of sulfated-proteoglycans in cartilage, it was found that 80 mM GlcN-S could inhibit s-GAG release around 64% when compared with IL-1β treated alone (figure 3.36 and table 3.2). While 1.0 μM sesamin could inhibit s-GAG release about 77%. The combination of 40 mM GlcN-S and 0.5 μM sesamin could inhibit s-GAG release around 41% and 80 mM GlcN-S and 1.0 μM sesamin inhibited this release about 62%. Thus, this result reveals that sesamin had no addtive effect with GlcN-S on inhibition of sulfated-proteoglycans degradation.

From these results (HA and s-GAG releases), it could not be summarized whether sesamin had additive effect with GlcN-S. Because of sesamin showed synergistic effect with GlcN-S on inhibition of HA release, but not on s-GAG release. Thus, we further investigated uronic acid remaining, due to uronic acid remaining could indicate the whole non-sulfated and sulfated proteoglycans in cartilage tissue.

As shown in figure 3.37 and table 3.3, 80 mM GlcN-S could inhibit uronic acid loss from cartilage around 7%, while 1.0 μM sesamin could inhibit about 5%. When the combination of 80 mM and 1.0 μM sesamin was used to treated cartilage, the inhibition of uronic acid loss was much increased (around 22%) when compared with those of the single treatment.

Altogether, it might be summarized that sesamin had additive effect with GlcN-S on inhibition of cartilage degradation.

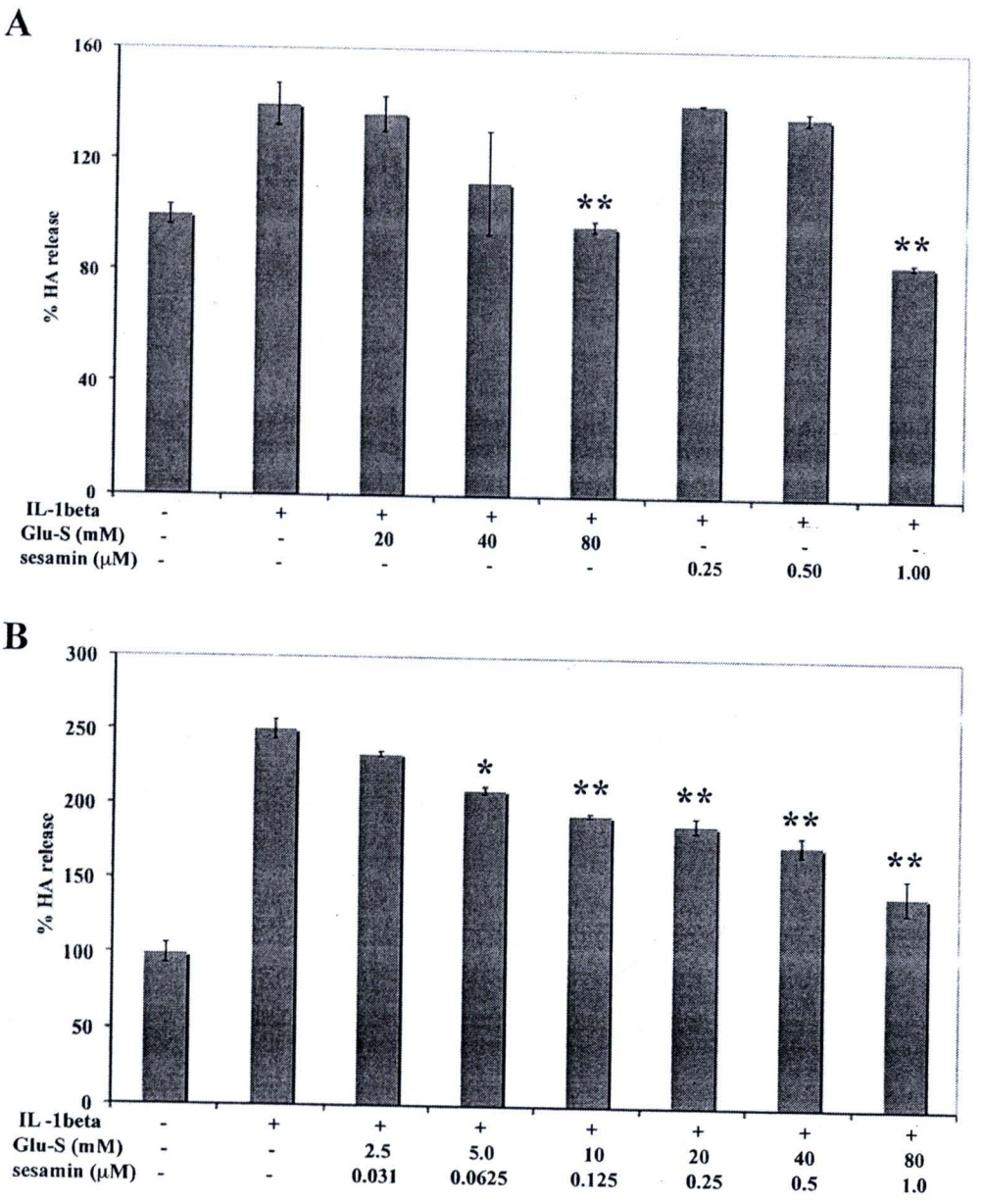

Figure 3.35 Effects of GlcN-S or sesamin on the release of HA (A), effect of GlcN-S and sesamin on the release of HA (B) from cartilage explant. Porcine cartilage explants were co-treated with 10 ng/ml IL-1β and various concentrations of GlcN-S and/or sesamin for 3 days. The conditioned media were analyzed for HA by ELISA test.

*, ** Denotes a value that is significantly different ($p<0.05$ and $p<0.01$, respectively) from the IL-1β control.

Glucosamin-sulfate (mM)	Sesamin (μM)	% HA reduction
20	-	3
40	-	28
80	-	43
-	0.25	0
-	0.50	4
-	1.00	56
2.5	0.031	17
5.0	0.0625	41
10	0.125	57
20	0.25	64
40	0.5	77
80	1.0	109

Table 3.1 The effect of GlcN-S and/or sesamin on the reduction of HA release from cartilage explant induced with IL-1β into media.

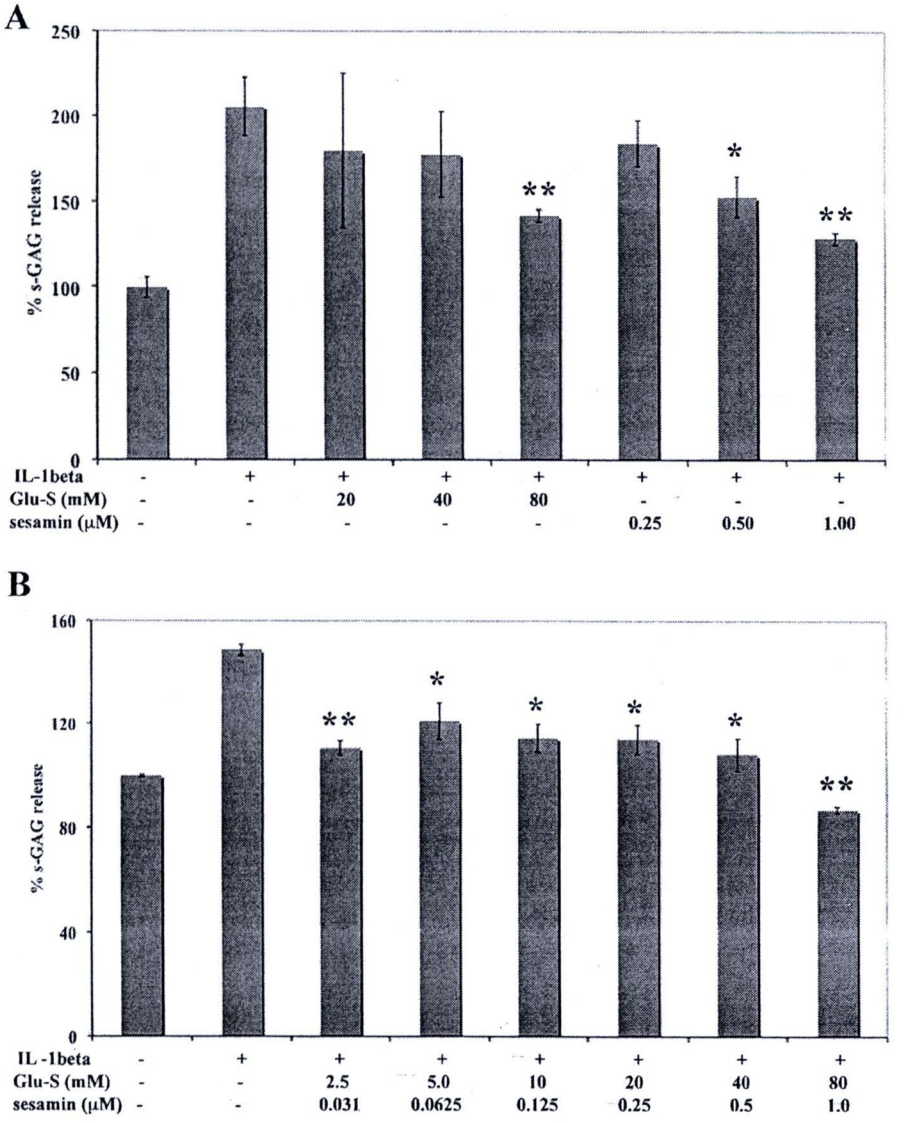

Figure 3.36 Effects of GlcN-S or sesamin on the release of s-GAG (A), effect of GlcN-S and sesamin on the release of s-GAG (B) from cartilage explant. Porcine cartilage explants were co-treated with 10 ng/ml IL-1β and various concentrations of GlcN-S and/or sesamin for 3 days. The conditioned media were analyzed for s-GAG by dye-binding assay test.

*, ** Denotes a value that is significantly different ($p<0.05$ and $p<0.01$, respectively) from the IL-1β control.

Glucosamin-sulfate (mM)	Sesamin (μM)	%s-GAG reduction
20	-	26
40	-	28
80	-	64
-	0.25	22
-	0.50	53
-	1.00	77
2.5	0.031	39
5.0	0.0625	28
10	0.125	35
20	0.25	35
40	0.5	41
80	1.0	62

Table 3.2 The effect of GlcN-S and/or sesamin on the reduction of s-GAG release from cartilage explant induced with IL-1β into media.

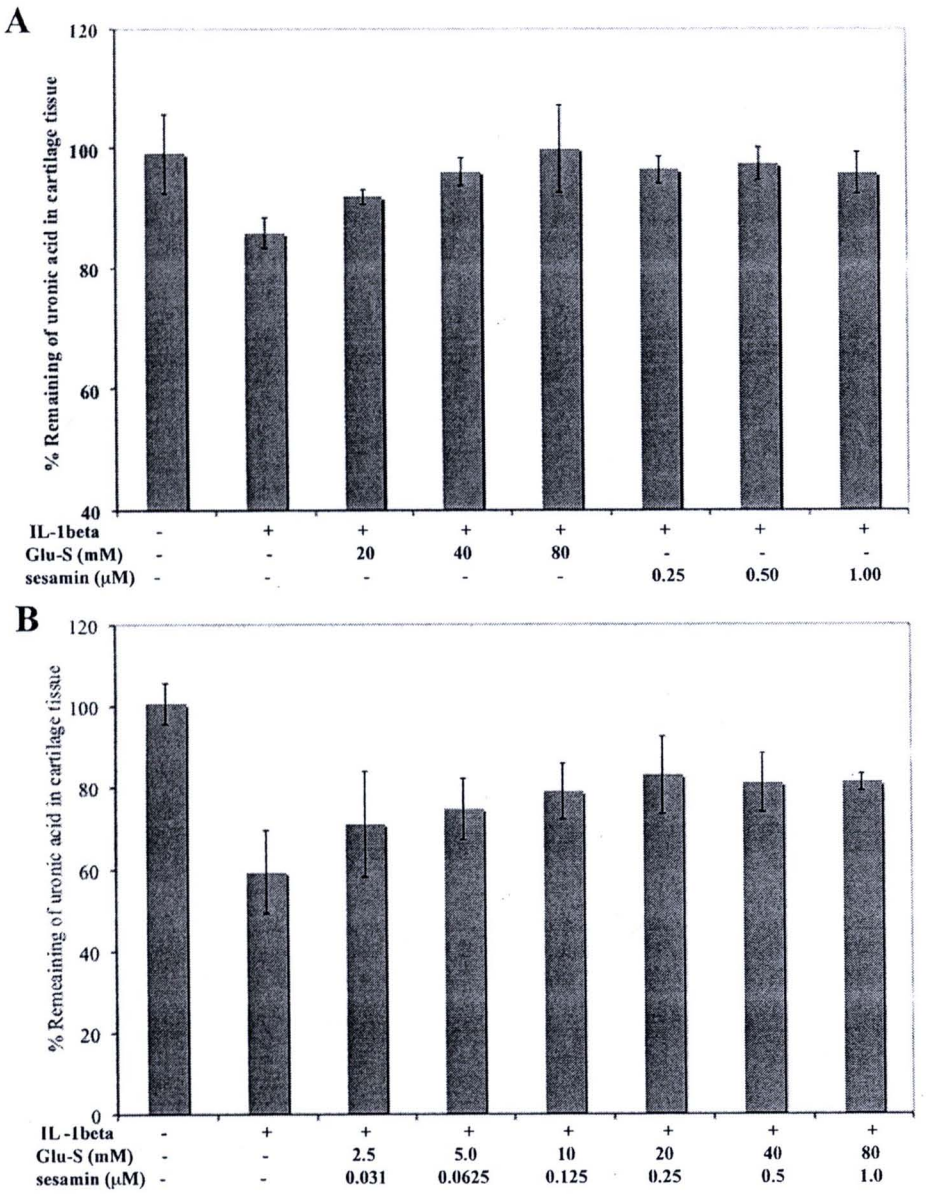

Figure 3.37 Effects of GlcN-S or sesamin on the remaining of uronic acid (A), effect of GlcN-S and sesamin on the remaining of uronic acid (B) in cartilage tissue. Porcine cartilage explants were co-treated with 10 ng/ml IL-1β and various concentrations of GlcN-S and/or sesamin for 3 days. The conditioned cartilage discs were digested with papain and then measured uronic acid remaining using colorimetric method.

Glucosamin-sulfate (mM)	Sesamin (μM)	% inhibition of uronic acid loss
20	-	3
40	-	5
80	-	7
-	0.25	5
-	0.50	6
-	1.00	5
2.5	0.031	12
5.0	0.0625	16
10	0.125	20
20	0.25	24
40	0.5	22
80	1.0	22

Table 3.3 The effect of GlcN-S and/or sesamin on the reduction of uronic acid loss from cartilage explant induced with IL-1β.