CHAPTER IV

DISCUSSIONS AND CONCLUSIONS

Osteoarthritis (OA) is the most common form of arthritis, affecting millions of people worldwide. It remains the major cause of disability in the elderly, affecting about 60% of men and 70% of women above the age of 65. OA is a multifactorial degenerative joint disease in which the cartilaginous matrix of the articular joint is destroyed. Disruption of the balance between cytokine-induced catabolic and anabolic processes leads to cartilage erosion that can reach the level of subchonal bone. There were many evidence found that IL-1β was a key mediator of joint breakdown, through its ability to induce expression of several MMPs (259, 265). Synthesis of matrix component, is also down-regulated by IL-1β, including type II collagen, limiting the repair potential of articular cartilage (266-268). IL-1β has attracted the most interest as a target for OA disease modification. There were many studies investigated the effects of IL-1 receptor antagonist (IL-1Ra) and it was found that the addition of IL-1Ra to OA explants inhibits inflammatory mediators, MMPs production and increases type II collagen and aggracan synthesis (269, 270).

As regards therapeutic strategies for OA, there are a large number of active research and drug discovery programs aimed to identify structure-modifying ways to inhibit joint destruction in OA, and existing drug therapies to reduce symptoms. None of these approaches, however, has significant efficacy as a disease modifying anti-OA drug (271). Until recently, COX-2 inhibitors were widely used to provide

symptomatic relief, but the increased risk of heart attacks and strokes associated with these drugs led to the recall of some products from the market and warnings concerning their use (272, 273). As alternatives to non-steroidal anti-inflammatory drugs (NSAIDs) and COX-2 inhibitors, other symptom-modifying drugs currently in clinical trials for OA include nitric oxide-releasing analgesics, bradykinin B2 receptor antagonists and capsaisin analogues (274). Other treatments for OA could include intra-articular injection with long-acting corticosteroids or hyaluronan, which would also provide symptomatic relief. One recent alternative therapy is nutraceutical treatment. Glucosamine (GlcN) is becoming increasingly popular as an alternative treatment for OA.

Many phytochemicals from plants were studied and used as drug for many diseases for a long time until nowadays. It was interested to find out phytochemical, which can inhibit IL-1β effects for development as drug for OA treatment or at lease as combinatory medicine for OA drugs. Here, the two plants were interested. The first plant was *A. galanga* rhizome that was widely used in Thailand as a component in a hot compress to reduce pain or inflammation. Even there were many reports studied many effects of *A. galanga* as described in introduction part, however, there was no study concerning the active phytochemical from this part of plant. The second one was a phytochemical from the seeds of *S. indicum*. There were many reports as described in introduction part found that sesamin had the anti-inflammatory effect. However, the chondroprotective effect of this phytochemical was underinvestigated.

Thus here the chondroprotective effect of *A. galanga* rhizome extracts and sesamin were investigated and compared with each other. Moreover, the active phytochemical from *A. galanga* rhizome extract was also studied. The phytochemical,

which had the highest effect was chosen to study in the molar mechanism and in *in vivo* system, and moreover, to study its synergistic effect with glucosamine drug.

4.1 The chondroprotective effect of *Alpinia galanga* extracts and its active phytochemical

We found that all hexane, ethylacetate, acetone and methanol of *A. galanga* extracts had chondroprotective effects, with the acetone extract having the greatest activity. The active compound *p*-hydroxycinnamaldehyde, purified from the acetone fraction, inhibited the effects of IL-1β by reducing release of ECM molecules such as HA, s-GAGs and MMP-2. In addition, we demonstrated that *p*-hydroxycinnamaldehyde also reversed effects of IL-1β on both catabolic and anabolic gene expression by reducing expression of the catabolic genes, MMP-3 and MMP-13, and inducing expression of anabolic genes, collagen, SOX9 and aggrecan core protein.

4.2 The chondroprotective effect of sesamin

Sesamin showed chondroprotective effect by inhibition of the effects of IL-1β on HA, s-GAG and MMP-2 releases. Moreover, in HACs, sesamin could inhibit IL-1β effects on the mRNA expressions of MMP-1, MMP-13, type II collagen, SOX-9 and aggrecan core protein.

4.3 The comparison of chondroprotective effect between *p*-hydroxycinnamaldehyde and sesamin

As shown in details above that both *p*-hydroxycinnamaldehyde and sesamin had the chondroprotective effect. However, the ranges of effective doses of both

phytochemicals were different (*p*-hydroxycinnamaldehyde (20-160 μM) and sesamin (0.25-1.00 μM)). The effective doses of sesamin was lower than *p*-hydroxycinnamaldehyde. The highest dose of both *p*-hydroxycinnamaldehyde and sesamin could inhibit IL-1β effects around 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 to study more in the deep details including confirmation its condroprotective effect in long term porcine cartilage explant culture, molecular mechanisms of its chondroprotective effect and whether it work in animal model.

4.4 Chondroprotective effect of sesamin, its molecular mechanism and its effect in animal model

From the results in porcine cartilage explant, sesamin could inhibit the degradation of PGs and collagen. It was possible that sesamin could inhibit proteinase enzymes, which degrade PGs and collagen. As described above that 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 major collagen found in cartilage. Only MMPs including the classical collagenases (MMP-1, -8 and -13) and MT1-MMP can degrade fibrillar collagen (249). Thus, we further investigated how did sesamin could protect

PGs and collagen degradations.

The activities of aggrecanases were studied using specific antibody (BC-3) that can recognize revealed aggrecanase cleavage site on aggrecan core protein. It was found that IL-1β induced aggrecanase activities and sesamin could not revert this effect.

We thus focused on MMPs. The expressions of MMP-1, -3 and -13 were studied in HAC treated with IL-1β. IL-1β induced these MMPs both mRNA and protein levels. In the studying of protein levels of these enzymes, it also found that not only latent forms of these enzymes could be induced by IL-1β, but active forms were also induced. Interestingly, sesamin could inhibit the expressions both mRNA and protein levels of these three enzymes. Moreover, the activation of MMP-3 might be inhibited by sesamin.

The signal transductions in HAC induced by IL-1β was studied. We found that IL-1β induced the phosphorylations of all three MAPK protein families (p38, ERK1/2 and JNK) and also activated NFκB transcription factor. The activations of p38 and JNK were significantly inhibited by sesamin, but ERK1/2 was not and, moreover, NFκB activation also was inhibited by sesamin. Altogether, these results indicated that the condroprotective effects of sesamin might due to its abilities to revert some IL-1β signals in HAC leading to the inhibitions of MMP-1, -3 and -13 expressions.

The *in vivo* study was performed for investigation of sesamin effects, rats were induced to be OA using papain injection into the knee. After induction period, sesamin was injected directly into OA rats' knee. Papain injection led to pathological progressions of OA showing as disorganized cell zone and column and thinner

cartilage tissue when compared with normal rats' cartilage. Moreover, cartilage of papain-induced OA rats showed the losses of PGs and type II collagen from cartilage matrix, as indicated in Safranin O staining and type II collagen immunohistochemical staining, respectively. Sesamin could restrain these pathological progressions in the dose dependent manner. Amazingly, sesamin alone at the dose of 10 μM had ability to induced PGs and type II collagen in normal rats' cartilage tissue. Altogether, sesamin might be the good choice for OA treatment.

Because of there was no the report studied the level of sesamin in the joint after oral administration. Thus for oral administration level of sesamin to allow the 1-10 μM sesamin in the joint as the level by intraarticular injection was interested to further study by pharmacokinetic knowledge.

4.5 Chondroprotective effect of glucose derivatives and the additive effect of sesamin

There is evidence that GlcN is equally effective or even better in decreasing pain in patients with knee OA, as compare to low dose NSAID use (277, 278). Furthermore, there are several reports showing that there was less joint space narrowing in people with knee OA who took GlcN compared to placebo, over a period of 3 years (277, 278). This suggests that that GlcN can delay the progression of knee OA. In the last several decades, there has been an increasing number of patients who have started using GlcN, with or without direction from a physician. Although the effectiveness of GlcN has been debated in a recent article, there are clinical studies suggesting that GlcN probably has structure modifying effects in patients with knee OA (277, 278). The underlying effects of GlcN on cartilage that are responsible

for these clinical outcomes are still unclear. It has been proposed that addition of GlcN to chondrocyte cell cultures leads to more GAG production, since GlcN is the basic building block of GAG molecules. Some studies have supported this hypothesis (279-283). However, there are studies that observed negative effects on GAG production after GlcN addition (279-283). Apart from influencing matrix synthesis, several studies have shown that GlcN is also able to interfere with enzymatic matrix degradation (289-292). These conflicting results can have various causes.

It is unclear whether GlcN-S, Glc-HCl and N-acetyl-glucosamine (GlcN-Ac) have similar effects on cartilage. Differences in the results can also be explained by the varieties of culture models used (e.g., monolayer or pellet culture) and the variations in culture duration.

No previous studies have examined the metabolism of GlcN in humans. However, there is one published investigation of synovial and plasma GlcN concentrations in OA patients following oral administration of crystalline GlcN-S. That study reported that GlcN is bioavailable both systemically and at the site of action (the joint) (293). However, it is still unclear whether the administrated GlcN will be metabolized to Glc in humans, and whether metabolized Glc will have effects similar to those of GlcN. Thus, in this experiment, we studied and compared the effects of Glc, GlcA, GlcN-S and GlcN-HCl in two models using IL-1β to induce inflammation: first in a porcine cartilage explant model, and second, in a HAC culture model. In the porcine cartilage explant model, GlcN-S showed the highest chondroprotective effects (inhibited IL-1β's effect on HA and s-GAG degradation) followed by GlcN-HCl, while Glc and GlcA did not show these effects. In the HAC model, GlcN-S had the highest effect, shown by inhibition of IL-1β induced HA

release and MMP-2 activity, followed by GlcN-HCl and GlcA, but Glc had no effect. Thus in administration of glucose derivatives, if these reagents were metabolized to Glc, the metabolized compound might not have a chondroprotective effect.

There are also reports showing that GlcN decreases expression of both anabolic and catabolic genes in human OA cartilage explants (294). We expected that GlcN-S would show the highest inhibitory effect on IL-1β, because it had the highest effect in porcine cartilage explants. But our results showed that only MMP-13 gene expression could be reduced by GlcN-S. For the other catabolic gene, MMP-3 was mostly inhibited by GlcN-HCl, inversely, Glc, and GlcA further induced both MMP-3 and -13 expression in HAC treated with IL-1β. In anabolic gene expression, both AGG and SOX9 expression were not significantly changed due to IL-1β. AGG expression was induced by Glc and GlcA, but was reduced by GlcN-S and GlcN-HCl. SOX9 expression was increased by GlcA and decreased by GlcN-HCl. Altogether, it seemed that Glc and GlcA could induce both catabolic and anabolic gene expression while GlcN-S and GlcN-HCl reduced the expression of the catabolic genes.

The additive effect of sesamin with GlcN-S was also studied in porcine catilage explant treated with IL-1β. The degradation of PGs in cartilage tissue was investigated. The degradation of non-sulfated PG (HA) and sulfated PGs (s-GAG) were measured and found that sesamin showed additive effect with GlcN-S on inhibition of non-sulfated PG degradation, but not on sulfated-PGs degradation. However, the whole PGs remaining were measured by measuring uronic acid remaining in conditioned cartilage. Sesamin showed additive effect with GlcN-S on inhibition of PGs loss from cartilage tissue. Altogether, sesamin showed additive effect with GlcN-S on inhibition of PGs loss from inflamed cartilage.

SUMMARY

Our study of chondroprotective effect of phytochemicals for further development of OA drug or as combined medicine for OA treatment, sesamin was the best phytochemical for this purpose. Sesamin itself had anti-arthritic effect both in *in vitro* and *in vivo* systems and, moreover, had additive effect with GlcN-S to protect cartilage degradation. Altogether, sesamin was a good candidate to develop for OA treatment.

For the comparison of tested phytochemicals and chemicals which were used in this study, it could be ordered the tested chemical according of their chondroprotective effect as follow: (1) the combination of GlcN-S and sesamin, (2) sesamin, (3) *p*-hydroxycinnamaldehyde, and (4) GlcN-S.

FURTHER STUDIES

1. In all of our experiment, sesamin was used to treated or injected directly into cartilage or joint. Due to there was the metabolism of sesamin after oral administration and some of it may metabolized to enterolactone. Thus it is interested to use pharmacological technique to deliver sesamin into the synovial joint without metabolism after oral administration or after application on the skin.
2. The clinical trial in patients with OA is in further process of our study.