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Stigmasterol blocks cartilage degradation in rabbit model of osteoarthritis

Wei-Ping Chen, Chong Yu, Peng-Fei Hu, Jia-Peng Bao, Jing-Li Tang and Li-Dong Wu✉

Department of Orthopedics Surgery, the 2nd Affiliated Hospital of Medical College, Zhejiang University, Hangzhou, People's Republic of China

Stigmasterol has been shown exihbit anti-osteoarthritic properties *in vitro* studies. However, the *in vivo* effects of stigmasterol on cartilage are still unclear. This study investigated the anti-osteoarthritic properties of stigmasterol on cartilage degradation in a rabbit model of osteoarthritis (OA). Twenty rabbits underwent bilateral anterior cruciate ligament transection (ACLT) to induce OA. Five rabbits were used as normal control. Two weeks after operation, the rabbits were randomly divided into two groups. Each group of 10 rabbits received intra-articular injection with 0.3 ml of stigmasterol in left knees and vehicle in right knees, once weekly. Group 1 was killed 6 weeks after ACLT and 2 were sacrificed 9 weeks after ACLT. The knee joints were assessed by gross morphology, histology and gene expression analysis. We found that expression of genes encoding matrix metalloproteinases (MMPs) was significantly higher while tissue inhibitors of metalloproteinase (TIMP)-1 was significantly lower in the both joints of the two OA groups compared to normal contrals. Stigmasterol reduced the cartilage degradation as assessed by histological analysis and markedly suppressed MMPs expression both in group 1 and group 2. Our results suggest that stigmasterol may be considered as a possible therapeutical agent in the treatment of OA.

Key words: matrix metalloproteinases, osteoarthritis, stigmasterol

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INTRODUCTION

Osteoarthritis (OA) is a chronic joint disease manifested by cartilage degradation along with severe joint pain. Chondrocytes exposed to abnormal stimuli such as mechanical damage or proinflammatory stimuli including interleukin-1 (IL-1) and tumor necrosis factor α (TNF-α), release matrix metalloproteinases (MMPs), the main matrix-degrading enzymes, leading to the breakdown of extracellular matrix (ECM) (Rowan *et al*., 2008). MMP-13 is well known as the most effective proteinase because of the ability to cleave collagen II which is the main component of ECM (Billinghurst *et al*., 1997).

Treatment of OA is still a problem. Although nonsteroidal anti-inflammatory drugs and selective cyclooxygenase-2 (COX-2) inhibitors are effective, they have severe side effects such as gastrointestinal bleeding and cardiovascular diseases (Farkouh *et al*., 2004; Lazzaroni *et al*., 2004). There is still a need to investigate new anti-arthritic agents.

Phytosterols are plant lipids whose chemical structure is similar to that of cholesterol. Previous studies have shown that phytosterols exert various biological effects such as anti-cancer, anti-pyretic and immune-modulating activities (Gupta *et al*., 1980; Raicht *et al*., 1980; Bouic *et al*., 1996; Bouic *et al*., 1999). In addition, their anti-inflammatory activities are also well demonstrated (Navarro *et al*., 2001; Okoye *et al*., 2010; Youssef *et al*., 2010). The role of phytosterols in rheumatoid arthritis (RA) has been investigated previously (Thiers, 1953). Recently, Gabay et al demonstrated that stigmasterol, a phytosterol, exerted anti-osteoarthritic properties in mouse chondrocytes and human chondrocytes (Gabay *et al*., 2010). That was the first study to report a possible role of phytosterols in preventing OA. However, the *in vivo* effects of stigmasterol on cartilage are still unclear. Therefore, in the present study, we investigated the *in vivo* effects of stigmasterol on cartilage in experimental OA.

MATERIAL AND METHODS

Reagents. Stigmasterol was purchased from Sigma-Aldrich (St.Louis, MO, USA). Stigmasterol stock solution at a concentration of 20 mg/ml was prepared in ethanol. Then, the solution was diluted with 50% polyethylene glycol (PEG).

OA induction and animal treatment. With the approval by the Institutional Animal Care and Use Committee of Zhejiang University (Hangzhou, China), twenty-five New Zealand White rabbits (male, 2.0–2.5 kg) were used in this study. Twenty rabbits underwent bilateral anterior cruciate ligament transection (ACLT) as described previously (Yudoh *et al*., 2007). Five rabbit were used as normal control. Two weeks after operation, the rabbits were randomly divided into two groups. Both groups of 10 rabbits were intra-articularly injected with 0.3 ml of stigmasterol solution (20 μg/ml in PEG) in left knees and vehicle in right knees, once weekly. Group 1 was killed 6 weeks after operation and 2 were sacrificed 9 weeks after operation. The knee joints were assessed by gross morphology, histology and gene expression analysis.

Macroscopic evaluation. Scoring of the articular cartilage of the rabbit knee joints was performed in a blind manner after using India ink. The criteria used were as follows: grade 1 (intact surface), grade 2 (minimal fibrillation), grade 3 (overt fibrillation), grade 4 (erosion) (Yoshioka *et al*., 1997).

✉e-mail: ldwu@yahoo.com

Abbreviations: ACLT, anterior cruciate ligament transection; COX-2, cyclooxygenase-2; DMOAD, disease modifying OA drug; ECM, extracellular matrix; EDTA, ethylenediaminetetraacetate; IL-1, nterleukin-1; MMPs, matrix metalloproteinases; OA, osteoarthritis; PEG, polyethylene glycol; RA, rheumatoid arthritis; TIMP, tissue inhibitors of metalloproteinase; TNF-α, tumor necrosis factor α.

Table 1. Criteria for histologic evaluation

Stage = % Involvement (surface, area, volume)
0 = No OA activity seen
1 = 10%
2 = 10-25%
3 = 25-50%
4 = >50%
Grade = OA depth progression into cartilage
0: surface intact, cartilage intact
1: surface intact
2: surface discontinuity
3: vertical fissures
4: erosion
5: denudation
6: deformation

Histological assessment. Femoral condyles from both knees were fixed in 10% neutral buffered formalin, decalcified with buffered ethylenediaminetetraacetate (EDTA) and dehydrated in a series of ethanol solutions. Then, the cartilage was embedded in paraffin, cut into 5-μm sections. The sections were stained with Safranin O-fast green. The OARSI assessment system with a combined score of grade (0–6) × stage (0–4) was used to evaluate the sections in a blind manner (Pritzker *et al.*, 2006).

Gene expression analysis of cartilage. Cartilage was pulverized in liquid nitrogen, and total RNA was extracted using TRIzol reagent (Invitrogen). For the first strand cDNA synthesis, 1 μg of RNA was reverse transcribed using Moloney murine leukemia virus reverse transcriptase cDNA synthesis kit (Promega, USA) at 37°C for 1 h. Gene expression of MMP-1, MMP-3, MMP-13 and tissue inhibitors of metalloproteinase (TIMP-1) was quantitated by real-time quantitative PCR with the iCycler apparatus system (Bio-Rad, USA). The primers used were shown in Table 2. 18S rRNA was used as an internal control. Levels of target gene expression were calculated as follows: $n=100\times2^{-}$ (ΔCT targeted gene –ΔCT 18S rRNA).

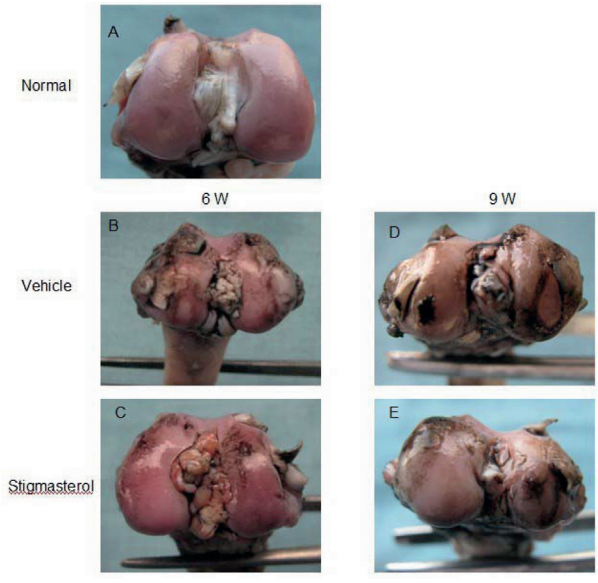

Figure 1. Gross appearance of femoral condyles
Gross morphology of femoral condyles from normal **(A)** and ACLT rabbits 6 and 9 weeks after operation. Femoral condyles in vehicle-treated group showed more serious lesions during the development of OA, while the lesions in stigmasterol-treated cartilage was less severe 6 weeks (**B** compare with **C**) and 9 weeks (**D** compare with **E**) after ACLT. OA = osteoarthritis; ACLT = anterior cruciate ligament transection.

Statistical analysis. All data were expressed as mean ± standard deviation (S.D.). Statistical analyses were performed with, SPSS 12.0 software for Windows. Statistical analysis of the gross morphology was performed by non-parametric Mann-Whitney U test, histological and gene expression data was analyzed by a paired *t*-test. *P* values less than 0.05 were considered significant.

RESULTS

Macroscopic grading of cartilage

The articular cartilage showed various severities of lesions. Cartilage from OA animal treated with vehicle showed significant severe lesions compared to normal cartilage. Those lesions were more severe in cartilage 9 weeks after ACLT than 6 weeks after ACLT. The stigmasterol-treated animal showed less severe cartilage lesions compared with the vehicle-treated group, both in 6 and 9 weeks after ACLT, however, the difference was not significant ($P>0.05$, data not shown). Figure 1 presents the cartilage lesions in different groups.

Histological evaluation

Representative histological pictures of control, vehicle-treated OA and stigmasterol-treated OA cartilage are presented in Fig. 2, and the histological scores are shown in Table 3. In vehicle-treated knees, ACLT led to cartilage degradation. Surface irregularity and depletion were noted in the cartilage 6 weeks after ACLT. Severe degradation such as cartilage erosion was observed 9 weeks after ACLT. The cartilage degradation was significantly diminished by stigmasterol.

Gene expression in cartilage

There was a significant increase in the expression of MMP-1, MMP-3 and MMP-13 genes in the cartilage from vehicle-treated OA animal cartilage harvested 6 or 9 weeks after ACLT compared to normal cartilage. In contrast, expression of the TIMP-1 gene was decreased. In addition, the MMPs expression increased in parallel with the progression of OA. Furthermore, stigmasterol

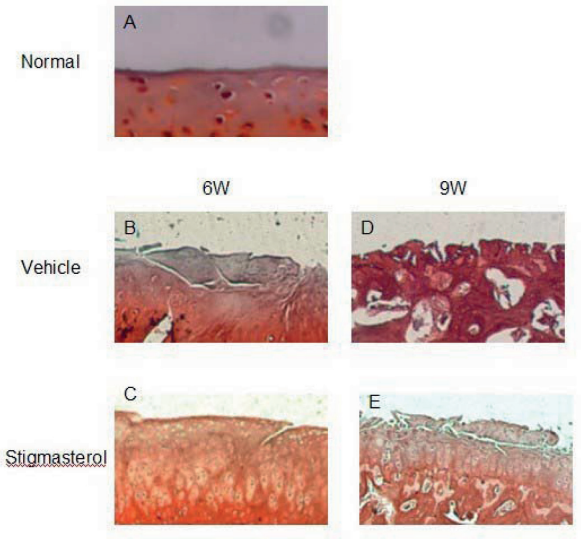

Figure 2. Safranin-O staining of articular cartilage
Safranin O staining was performed in sections of cartilage. Typical cartilage lesions are shown (Original magnification ×50).

Table 2. Primers for targeted genes

Targeted genes	Sequence (5′-3′)*	Amplicon length (bp)	Accession number
MMP-1	S: GACGTGGACCGACAACAGTGA A: GGGGAACATTAGTGCTCCTACATC	112	AH005676
MMP-3	S: ACACCGGATCTGCCAAGAGA A: CTGGAGAACGTGAGTGGAGTCA	89	NM001082280
MMP-13	S: TTGACCACTCCAAGGACCCAG A: GAGGATGCA GACGCCAGAAGA	252	NM001082037
TIMP-1	S: CAACTGCGGAACGGGCTCTTG A: CGGCAGCGTAGGTCTTGGTGAA	102	AY8297305
18S rRNA	S: GACGGACCAGAGCGAAAGC A: CGCCAGTCGGCATCGTTTATG	119	EU236696

*S = Sense; A = Antisense

significantly decreased the expression of the MMP-1, MMP-3 and MMP-13 genes, and increased the expression of TIMP-1 compared to the vehicle-treated group (Fig. 3).

DISCUSSION

Current treatment of OA is unsatisfactory and although symptom relief, no disease modifying OA drug (DMOAD) is yet available. There is still a need to find new agents for the treatment of OA.

Recently, Gabay *et al.* (2010) demonstrated that stigmasterol, a phytosterol, showed anti-osteoarthritic properties in both mouse chondrocytes and human chondrocytes in response to IL-1β. Little is known about the *in vivo* effects of stigmasterol on OA. Therefore, we design this study to investigate whether stigmasterol exerted *in vivo* effects on cartilage degradation in experimental OA. In the experimental rabbit OA model induced by ACLT, treatment with stigmasterol reduced the progression of cartilage damage as assessed by morphological non-significantly and histological evaluation. In addition, treatment with stigmasterol resulted in the down-regulation of expression of MMP-1, MMP-3, MMP-13 and up-regulation of TIMP-1. These findings confirmed the *in vitro* effects of stigmasterol reported by Gabay and coworkers.

It is well established that numerous proteinases such as MMPs, aggrecanases and cathepsins are implicated in the catabolism of OA cartilage (Dean *et al.*, 1989; Konttinen *et al.*, 2002; Tortorella *et al.*, 2001). Among them, MMPs are believed to play pivotal roles in cartilage matrix degradation. MMP-13 has received extensive attention because it is mainly responsible for type II collagen degradation. An elevated MMP-13 expression was noted in arthritic cartilage (Aigner *et al.*, 2001). In addition, a selective MMP-13 inhibitor has been shown to reduce cartilage degradation in experimental OA induced by ACLT in rabbits and in the rat model of mono-iodoacetate (MIA)-induced OA (Johnson *et al.*, 2007; Baragi *et al.*, 2009). In the present study, we focused on changes of gene expression of MMPs in cartilage due to their importance in OA. The present results revealed that the expression of MMP-1, MMP-3 and MMP-13 was significantly elevated by ALCT compared to normal cartilage and this up-regulation was related to the cartilage degradation in the progression of OA. Our results are partly consistent with the findings reported by Wu and coworkers (Wu *et al.*, 2008), who demonstrated that the level of MMP-1 expression was correlated with the degree of cartilage degradation in ACLT rabbits. In addition, we found that the expression of TIMP-1 was decreased along with the cartilage degradation. Thus, the balance between MMPs and TIMPs was disturbed. Since the imbalance between MMPs and TIMPs in the present study increased with the progressive cartilage degradation as assessed by morphology and histology, our results confirmed the important role of MMPs/TIMPs in the process of OA.

In the present study, treatment with stigmasterol by intra-articular injection resulted in the down-regulation of the expression of MMP-1, MMP-3 and MMP-13 and up-regulation of TIMP-1 as compared to the vehicle-treated groups. These observations suggest that stigmasterol administration regulated the balance between MMPs and TIMPs during the progression of OA. This could be partly supported by the finding that stigmasterol inhibited the gene expression of MMP-3 and MMP-13 in mouse chondrocytes and human chondrocytes *in vitro* (Gabay *et al.*, 2010). An inhibition of MMPs by phytosterol was also observed in another system as reported by Grether-Beck *et al.* (2008). In addition, a preparation rich in phytosterol, named avocado-soybean unsaponifiables (ASU), also has been shown to inhibit MMPs expression in experimental OA in dogs (Boileau *et al.*, 2009). These observations suggest that phytosterol may exert inhibitory effects on MMPs in different systems. Additionally, many

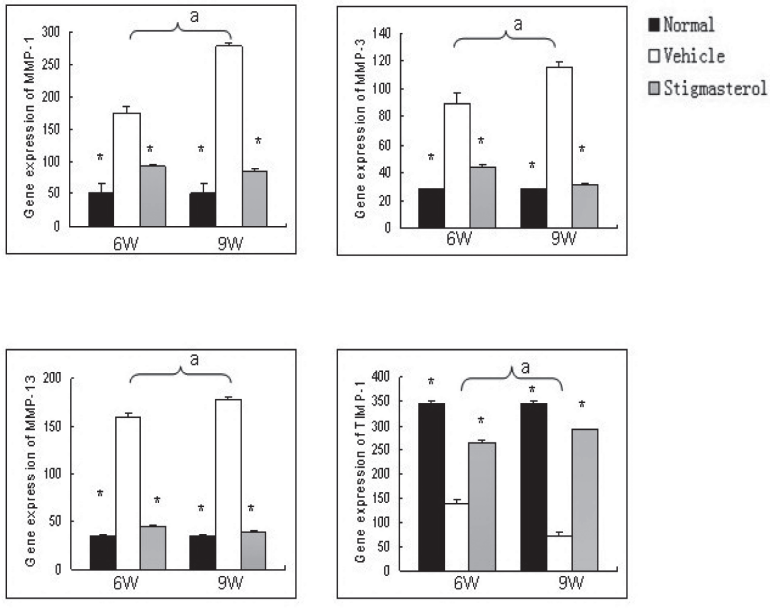

Figure 3. Gene expression of MMP-1, MMP-3, MMP-13 and TIMP-1 in cartilage
Quantitative real-time PCR was used to analyze the expression of MMP-1, MMP-3, MMP-13 and TIMP-1 genes in cartilage. Data are presented as mean ±S.D. $^{a}P<0.05$. *$P<0.05$ compared to the vehicle-treated group.

Table 3. Histological assessment of articular cartilage using OARSI assessment system

	Vehicle	Stigmasterol
6 weeks after ACLT	5.8±2.05	2.8±1.1*
9 weeks after ACLT	13.6 ±2.19	9.6±2.5*

Data are presented as mean ± S.D. *P<0.05.

agents showing inhibitory effects on MMPs have shown beneficial effects in experimental OA. Thus, we speculate that the beneficial effects of stigmasterol on cartilage may be partly associated with the regulation of the MMPs/TIMPs system.

The main limitations of our study are its duration (6 or 9 weeks after ACLT) and the single dosage in the study. Longer time could provide more information about the changes of gene expression of MMPs and TIMPs as well as the *in vivo* effects of stigmasterol in experimental OA. For instance, in earlier studies, three stage of OA was used to investigate the gene expression of catabolic mediators in ACLT rabbits (Mehraban *et al*., 1997; Bao *et al*., 2009). In addition, we only used one dosage in the experiment, maybe different dosage could better illustrate the effects of sigmasterol. Another limitation is that the mechanism by which stigmasterol regulated the MMPs/TIMPs in cartilage *in vivo* remains unclear. *In vitro*, stigmasterol has been shown to inhibit pro-inflammatory cytokines and matrix degradation mediators partly via inhibiting the nuclear factor-κB (NF-κB) pathway (Gabay *et al*., 2010). The effects of stigmasterol on another important signalling pathway, mitogen-activated protein kinase (MAPK) pathway which is implicated in MMPs regulation remains unknown (Yan & Boyd, 2007). In addition, cartilage degradation is partially dependent on MMPs activities, there are mechanisms involved in cartilage degradation indepent on MMPs, and the effects of sigmasterol on thes mechanisms are also unclear. Therefore, further studies are needed to get a better understanding about the mechanisms of action of stigmasterol in OA.

In summary, we first reported that stigmasterol effectively inhibits cartilage degradation in a rabbit ACLT model. Our results suggest that stigmasterol may be considered as a possible therapeutical agent in the treatment of OA. Further studies are needed to elucidate the action mechanism of the regulation of MMPs by stigmasterol.

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Competing interests

The author(s) declare that they have no competing interests.

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