Expression level of proteoglycan, collagen and type II collagen in osteoarthritis rat model is promoted and degradation of cartilage is prevented by glucosamine methyl ester

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Abstract. – OBJECTIVE: In the current study, the effect of glucosamine methyl ester on cartilage degeneration in osteoarthritis rat model was investigated.

MATERIALS AND METHODS: Forty Sprague-Dawley rats were assigned into 5 groups of 8 animals each. Osteoarthritis was induced in 4 groups using medial parapatellar incision followed by anterior cruciate ligament transection and meniscectomy. Normal and model osteoarthritis groups were given normal saline. The three treatment groups received 2, 5 and 10 mg/kg doses of glucosamine methyl ester daily for one month.

RESULTS: Microscopic examination of the knee cartilage showed a significant reduction in degeneration score in the treatment groups. Enzyme-linked immunosorbent assay revealed inhibition of interleukin-1β expression and nitric oxide generation on treatment with glucosamine methyl ester. Expressions of matrix metalloproteinase-3 and -13 in the treatment groups were significantly lower compared to the model osteoarthritis group. Polymerase chain reaction revealed an increased expression of tissue inhibitor of metalloproteinases 1 on treatment of rats with glucosamine methyl ester. In the osteoarthritis rats treated with various doses of glucosamine methyl ester staining, the level of toluidine blue and Masson’s trichrome increased. In addition, the level of type II collagen was also higher in the rats of treatment group. The level of proteoglycan, collagen and type II collagen in OA rats treated with 10 mg/kg doses was ~3.2- (p<0.01), 2.4- (p<0.02), and 3.6- (p<0.05) fold, respectively higher compared to the untreated animals.

CONCLUSIONS: Glucosamine methyl ester, therefore, prevents degeneration of cartilage in osteoarthritis rats. It exhibits its effect by promoting proteoglycan, collagen, type II collagen, tissue inhibitor of metalloproteinases 1, and decreasing matrix metalloproteinase. Therefore, glucosamine methyl ester exhibits therapeutic effect against osteoarthritis.

Key Words: Degeneration, Osteoarthritis, Metalloproteinase, Meniscectomy, Proteoglycan.

Introduction

Osteoarthritis is characterized by articular cartilage degeneration through apoptosis of chondrocytes and breakdown of components of extracellular matrix. This joint disorder is found commonly in adults and involves the formation of osteophyte, remodeling of subchondral bone, and inflammation. Studies have demonstrated that mechanism of osteoarthritis is very complicated, consisting of several interconnected factors such as the breakdown of proteoglycan, proteolysis of collagen, and subsequent degeneration of cartilage. Degradation of components of cartilage extracellular matrix is caused by various factors such as reactive oxygen species, interleukin-1β, and matrix metalloproteinases. Since matrix metalloproteinases catalyze the breakdown of cartilage extracellular matrix, therefore, inhibition of their expression is considered to be of therapeutic importance for osteoarthritis treatment. Chemotherapeutic agents which inhibit inflammatory processes are also beneficial in osteoarthritis prevention.

Glucosamine is one of the amino monosaccharides which is presented as glycosaminoglycan in the tissues of cartilage where it is responsible for maintenance of strength and elasticity. Reports involving osteoarthritis animal models have shown that treatment with glucosamine prevents degradation of the cartilage and leads to expression of proteoglycans. After successful clinical trials, glucosamine has been used for the treat-
ment of osteoarthritis in human beings from very long time\textsuperscript{15-18}. Researches\textsuperscript{19-21} demonstrated that glucosamine treatment modifies various symptoms of osteoarthritis. It has also been shown that production of inflammatory factors, such as reactive oxygen species and interleukins, is suppressed by glucosamine\textsuperscript{22}. The role of glucosamine in the prevention of cartilage degradation and osteoarthritis symptom relieving is suggested by the Osteoarthritis Research Society International\textsuperscript{23}. The present study demonstrates the role of modified glucosamine, glucosamine methyl ester in the osteoarthritis treatment in a rat model. Several factors were analyzed in the osteoarthritis rats after glucosamine methyl ester treatment. These factors include proteoglycan, collagen, type II collagen, matrix metalloproteinases, tissue inhibitor of metalloproteinases 1, nitric oxide, and interleukin-1β. The results demonstrated that glucosamine methyl ester efficiently prevents development and progress of osteoarthritis in the rats and, thus, could be used for its treatment.

Materials and Methods

Preparation of OA Rat Model and Treatment Strategy

Sprague-Dawley rats 40 in number and 180-220 g in body weight were purchased from the Laboratory Animal Center, Guangzhou University of Traditional Chinese Medicine Guangzhou, China. The animals were obtained under license no. scxk 2016-0123. The rats were housed at a temperature of 20°C under humidity-controlled conditions and were provided free access to the standard laboratory food ad libitum and water. The study was approved by the Committee for Animal Care and Use, Guangzhou University of Traditional Chinese Medicine (2016-B345). Each rat was injected with 30 g/l of pentobarbital anesthesia through a marginal ear vein. OA was induced in 32 rats. The protocol involved a medial parapatellar incision into the knee skin of left side hind limb. The animals were subsequently subjected to anterior cruciate ligament transection\textsuperscript{24-26} and meniscectomy for the induction of knee OA. Rats in the control group were subjected to medial parapatellar incision alone. All the rats were given prophylaxis for one week after surgery through the intramuscular route. The 32 OA rats were divided into four groups of 8 animals each; untreated control, low dose, medium dose, and high dose groups. The untreated control and sham-operated groups received 0.9% normal saline. Animals in the low, medium, and high dosage groups were given 2, 5 and 10 mg/kg doses of glucosamine methyl ester daily for one month intraperitoneally, respectively.

Examination of Cartilage's Pathological Alteration

The rats were sacrificed after completion of the treatment by air embolism to extract the knee joints of left side hind limb. The joints were fixed for 24 h in 4% paraformaldehyde immediately after isolation. EDTA (10%) solution was used for decalcification of the tissues over a period of 3 months. For decalcification, the EDTA solution was replaced every 3 days. The decalcified tissues were then paraaffin-embedded and subsequently sliced into thin 2 µm sections. The tissues were examined for histological changes using hematoxylin and eosin stain. A TS100 1x70 inverted-phase contrast microscope (Nikon Inc., Melville, NY, USA) was used for observing the stained tissue sections. The changes in cartilage histology were described using the scale of Peletier et al\textsuperscript{27}. Colorless cartilage with a smooth surface was assigned score- 0, malacic and smooth cartilage was assigned score- 1, a thin cartilage that appeared similar to bundle of fibre was scored- 2, the cartilages that appeared like fibrous bundle were scored- 3, cartilages with prominent degradation were given score- 4.

Nitric Oxide and Interleukin-1β Level Analysis

Normal saline (1 mL) was injected into the cavity of the knee joint of all the studied rats. The fluid from the joints was then collected into a test tube and subsequently analyzed for the presence of nitric oxide and interleukin-1β level using the enzyme-linked immunosorbent assay kit (BD Biosciences, Franklin Lakes, NJ, USA). The fluid was microfuged for 10 min at 1,500 rpm and, then, frozen at a temperature of -30°C before analysis using ELISA. The fluid was put into the 96-well microplates and treated with hydrogen peroxide and tetramethylbenzidine. A DNM-9606 microplate reader (Perlong Medical, Beijing, China) was used for the measurement of the optical density for each of the well at 450 nm.

Western Blot Assay for Determination of MMP-3 and MMP-13 Protein Expression

The cartilage samples from the rats were sliced into thin 2 µm sections and, then, were put into the homogenizer. The homogenized tissue
samples were treated with the TRIzol reagent (BD Biosciences, Franklin Lakes, NJ, USA) and phenylmethanesulfonyl fluoride, 1 ml and 40 µl, respectively for 15 min at 0°C. The samples were then transferred into the Eppendorf tubes and were maintained for 1 h at 0°C, centrifuged for 20 min at 12,000 x g to collect the supernatant. The bicinchoninic acid assay was used for the measurement of the concentration of total proteins in the supernatant. The proteins were separated by electrophoresis using sodium dodecyl sulphate-polyacrylamide gel (12%). The proteins were transferred onto the polyvinylidene fluoride membrane at room temperature for 1 h. The non-specific sites in the membranes were blocked by incubation for 1 h at room temperature with skimmed milk (5%). The incubation of the membranes was performed for overnight at 4°C with antibodies against matrix metalloproteinase-3 (sc-1719R), matrix metalloproteinase-13 (sc-2119), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH; dilution, 1:500; all obtained from BD Biosciences). The membranes were washed and subsequently subjected to 1 h incubation at room temperature with anti-rabbit secondary antibodies (563-2; dilution, 1:1,000; Zemai Biotech Corporation, Shanghai, China). The complexes were observed using an enhanced chemiluminescence detection system (LAS MINI 4000, GE Healthcare Life Sciences, Little Chalfont, UK).

Quantitative Polymerase Chain Reaction (qPCR) for Analysis of Tissue Inhibitor of Metalloproteinases 1 (TIMP1) mRNA Expression

The alteration in the TIMP1 expression was analyzed by the measurement of mRNA levels. The TRIzol reagent (BD Biosciences, Franklin Lakes, NJ, USA) was used for isolation of total RNA from the rat cartilage. The extracts of the cartilage tissues were obtained after treatment with NP-40 lysis buffer (0.1% NP-40, 150 mM sodium chloride, 1 mM ethylene diamine tetraacetate, 50 mM Tris pH 8.0, 1 mM sodium vanadate, 1 mM phenylmethysulfonyl fluoride) containing protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA; P-8340). The concentration of the proteins in the extract was determined by the Bradford assay (Bio-Rad, Hercules, CA, USA). The proteins were resolved electrophoretically by loading equivalent amounts 10 µg on sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE; 6-10%) gels. The PrimeScript RT reagent kit (Sigma-Aldrich, St. Louis, MO, USA) was used for reverse transcription of RNA samples to obtain cDNA as per the manual protocol. The reaction mixture (10 ml) consisting of 5X PrimeScript buffer (2 µl), PrimeScript RT enzyme (0.5 µl), RNA sample (2 µl) and RNase-free dH2O (5 µl) was incubated for 20 min at 37°C followed by 20 sec heating at 90°C. The primers used for TIMP1 were GTC GCA TGC TGC GAG TTG AC, forward and GGG TGG CCA AGA GCC TTGT-reverse (IBSBSAOA-156; Sigma-Aldrich, St. Louis, MO, USA). The cDNA (2 µl) samples, 8.5 µl dH2O (8.5 µl) and SYBR Premix Ex Taq (12.5 µl) were mixed with 1 µl each of forward and reverse TIMP1 primers for qPCR analysis. The PCR procedure involved 40 cycles of denaturation for 5 min at 95°C, annealing for 30 sec at 60°C and extension for 20 sec at 72°C. After completion, a series of 71 cycles of 20 sec at 60-95°C were performed and the temperature was increased by 0.5°C after each cycle. The expression level of mRNA corresponding to GAPDH was taken as internal control. The primers for GAPDH mRNA were ACG TCCCAT CAC GAT CCTTC-forward and ACA CTC GGA TGA CGA ACT-reverse.

Statistical Analysis

All the data presented are mean ± standard deviation. The SPSS version 13.0 software for Windows (SPSS, Inc., Chicago, IL, USA) was used for statistical analysis. The Bonferroni test in one-way analysis of variance was used for pairwise comparison of multiple samples. For statistically significant differences p was considered as <0.05.

Results

Effect of Glucosamine Methyl Ester on Histological Changes in OA rat Cartilage

Histological examination of cartilage in normal control rat group showed smooth surface and absence of any other defect and, therefore, was assigned a score 0. In untreated OA rats, the cartilage appeared as bundle of fibers, had many cracks and prominent degradation getting a score of 4. Treatment of the OA rats with glucosamine methyl ester prevented cartilage degradation which was evident by increase in smoother cartilage surface with increase in treatment dosage. In the rats treated with 2, 5 and 10 mg/kg doses of glucosamine methyl ester, the scores were 3, 2 and 0, respectively (Figure 1).
Glucosamine Methyl Ester Decreases Nitric Oxide and Interleukin-1β Level in Joint Fluid of OA rats

In the untreated OA rats, the levels of nitric oxide and interleukin-1β were significantly (p<0.02) higher compared to the normal control group. The level of nitric oxide was 14.54 ± 2.0 and 6.0 ± 0.6 in the untreated OA and normal control rats, respectively. The interleukin-1β level in the untreated OA and normal control rats were respectively 16.23 ± 2.0 and 6.89 ± 0.5. In the OA rats, treatment with 2, 5 and 10 mg/kg doses of glucosamine methyl ester decreased the level of nitric oxide to 12.65 ± 1.55, 9.68 ± 1.2 and 6.98 ± 0.7, respectively. The level of interleukin-1β was decreased to 13.25 ± 1.59, 11.75 ± 1.0 and 7.61 ± 0.8, respectively in the OA rats treated with 2, 5 and 10 mg/kg doses of glucosamine methyl ester (Figure 2).

Figure 1. Glucosamine methyl ester prevents cartilage degradation in the OA rats. The OA rats were given 2, 5 and 10 mg/kg doses of glucosamine methyl ester daily for one month and then sacrificed to extract knee joint cartilage. A TS100 1x70 inverted-phase contrast microscope was used for examination of cartilage sections using hematoxylin and eosin staining. Magnification x250.

Figure 2. Effect of glucosamine methyl ester on nitric oxide and interleukin-1β content in OA rat knee joint fluid. The OA rats were treated with 2, 5 and 10 mg/kg doses of glucosamine methyl ester for one month daily and then sacrificed to collect the fluid. The content of nitric oxide and interleukin-1β in the fluid were determined by enzyme-linked immunosorbent assay. The presented data are the mean ± standard deviation for each of the rat group. *p<0.02 compared with normal group, #p<0.01, ##p<0.01 and ###p< 0.01 compared with 2, 5 and 10 mg/kg groups. nitric oxide = µmol/L and interleukin-1β = ng/L.
In osteoarthritis rat model degradation of cartilage is prevented by glucosamine methyl ester.

In osteoarthritis rat model degradation of cartilage is prevented by glucosamine methyl ester. A dose-dependent increase was observed in TIMP1 mRNA expression level. The expression of TIMP1 mRNA was found to be 0.39 ± 0.01, 0.51 ± 0.02 and 0.83 ± 0.03 in the rats treated with 2, 5 and 10 mg/kg doses, respectively compared to 0.29 ± 0.01 in the untreated rats. The expression of TIMP1 mRNA in the normal control group of rats was 0.89 ± 0.04.

Glucosamine Methyl Ester Decreases Expression of MMP-3 and MMP-13 Protein in OA Rats

The analysis of MMP-3 and MMP-13 protein expression in the cartilage tissues of the rats using Western blot assay showed the highest level in the untreated control group. In the OA rats treated with glucosamine methyl ester, the level of MMP-3 and MMP-13 protein expression was significantly decreased (p<0.02). The expression levels of MMP-3 and MMP-13 protein were reduced if compared to normal control group in the OA rats treated with 10 mg/kg doses of glucosamine methyl ester (Figure 3).

Glucosamine Methyl ester Increases mRNA Expression of TIMP1 in OA Rats

The RT-PCR analysis showed significantly (p<0.05) lower level of TIMP1 mRNA expression in untreated group of rats compared to the normal rats (Figure 4). In the rats treated with various doses of glucosamine methyl ester, a dose-dependent increase in TIMP1 mRNA expression level was observed. The expression of TIMP1 mRNA was 0.39 ± 0.01, 0.51 ± 0.02 and 0.83 ± 0.03 in the rats treated with 2, 5 and 10 mg/kg doses, respectively compared to 0.29 ± 0.01 in the untreated rats. The expression of TIMP1 mRNA in the normal control group of rats was 0.89 ± 0.04.

Glucosamine Methyl Ester Increases Proteoglycan, collagen and type II Collagen Level in the Cartilage of OA Rats

The cartilage staining using toluidine blue and Masson’s trichrome showed markedly higher level in the normal control compared to the untreated OA rat group (Figure 5). The expression level of type II collagen was also reduced in the untreated OA rats. In the OA rats treated with various doses of glucosamine methyl ester staining, the level of toluidine blue and Masson’s trichrome increased and level of type II collagen was also increased.

Figure 3. Decreases in expression of MMP-3 and MMP-13 protein in OA rats by glucosamine methyl ester. The expression of MMP-3 and MMP-13 protein in cartilage tissue sections of normal control and OA rats treated or untreated were analyzed using Western blot assay. The OA rats were treated daily for one month with 2, 5 and 10 mg/kg doses of glucosamine methyl ester. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as internal loading control. *p<0.05 and **p<0.05 compared with the untreated OA group.

Figure 4. The mRNA expression of tissue inhibitor of metalloproteinases 1 in OA rats is increased by glucosamine methyl ester. The OA rats were untreated or treated with various doses of glucosamine methyl ester for one and sacrificed to extract knee joint. The expression of TIMP-1 mRNA in cartilage tissues was analyzed using Quantitative polymerase chain reaction analysis. In the experiment, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as internal control. *p<0.05 and **p<0.05 compared with the untreated OA group.
higher. The levels of proteoglycan, collagen, and type II collagen in the OA rats treated with 10 mg/kg doses were ~3.2- \( (p<0.01) \), 2.4- \( (p<0.02) \) and 3.6- \( (p<0.05) \) fold higher in comparison with the untreated OA rats (Figure 5).

### Discussion

In UK and Netherland, osteoarthritis is found in more than 10% of the people in the age group of >55 years and one-fourth of such individuals are disabled severely\(^{28}\). In these patients, the knee joint articular cartilage is degenerated and the subchondral bone is also affected. Although the mechanism of osteoarthritis is not completely understood, it has been proposed that glycosaminoglycan-proteoglycan matrix has a main role in the development of osteoarthritis\(^{29}\). The current study was aimed to investigate the role of glucosamine methyl ester in the treatment of osteoarthritis in rat model. The research showed that glucosamine methyl ester prevented degradation of knee joint cartilage in osteoarthritis rats through inhibition of proteoglycan, collagen, and type II collagen reduction. Glucosamine methyl ester also reduced the content of nitric oxide and interleukin-\( \beta \) in the knee joint fluid in osteoarthritis rats. In the osteoarthritis rats, the treatment with glucosamine methyl ester reduced the level of MMP-3 and MMP-13 protein and promoted TIMP1 mRNA expression in cartilage tissues. Cartilage degradation in osteoarthritis animal models is commonly determined using a known protocol of scoring system\(^{27}\). In the current study, the scoring system showed prevention of cartilage degradation in the osteoarthritis rats on treatment with glucosamine methyl ester. Interleukin-\( \beta \), a pro-inflammatory cytokine, catalyzes the metabolism of chondrocytes leading to inhibition of proteoglycan formation\(^{30}\). Studies also revealed that interleukin-\( \beta \) promotes the formation of matrix metalloproteinase by synovial cells which then enhance the destruction of extracellular matrix in cartilage\(^{6}\). In addition, the expression of interleukins-6 and -8, as well as the formation of nitric oxide in chondrocytes, is facilitated by interleukin-\( \beta \)\(^{31}\). These factors play an important role in tissue inflammation and damage\(^{31,32}\). A higher level of nitric oxide inhibits expression of kinase C protein, which results in apoptosis of chondrocytes in the cartilage\(^{31}\). In the present study, the treatment of osteoarthritis rats with glucosamine methyl ester reduced the expression of interleukin-\( \beta \) and the production of nitric oxide in the cartilage tissues. Thus, glucosamine methyl ester inhibits inflammation of osteoarthritis rats through inhibition of interleukin-\( \beta \) expression and production of nitric oxide. Higher level of matrix metalloproteinase have been reported to induce cartilage degradation by decomposition of extracellular matrix\(^{34,35}\). The integrity of cartilage is maintained by the presence of type II collagen and proteoglycans which are degraded by the expression of matrix.
metalloproteinase. In the patients with joint disorder, the expression of matrix metalloproteinase is found to be higher in the synovial fluid. For the inhibition of matrix metalloproteinase expression, chondrocytes produce glycoproteins namely TIMPs. However, when there is imbalance in the ratio of matrix metalloproteinase and TIMPs, cartilage is degraded. The present work revealed that glucosamine methyl ester treatment of the osteoarthritis rats decreased the expression of matrix metalloproteinases-3 and -13. The expression of TIMP1 mRNA was increased in the cartilage tissues of osteoarthritis rats on treatment with glucosamine methyl ester.

Conclusions

We showed that glucosamine methyl ester prevented the degeneration of cartilage in osteoarthritis rats through up-regulation of proteoglycan, collagen and type II collagen expression, increase in TIMP1 mRNA level and decrease in matrix metalloproteinase. Therefore, glucosamine methyl ester could be used for the treatment of osteoarthritis.

Conflict of Interest

The Authors declare that they have no conflict of interest.

References


