

Original Research Article

Protective effect of glucosamine cyclohexyl ester on osteoarthritis in rat via targeting expressions of matrix metalloproteinase and tissue inhibitor of metalloproteinases-1

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Abstract

Purpose: To investigate the therapeutic effect of glucosamine cyclohexyl ester on osteoarthritis (OA) in a rat model.

Methods: Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and western blot assays were used to analyze the effect of glucosamine cyclohexyl ester on changes in mRNA and protein expressions of matrix metalloproteinase and tissue inhibitor of metalloproteinases-1 in isolated rat chondrocytes, and in a rat model of OA. The rat model of OA was prepared by injecting monoiodoacetate to Sprague-Dawley rats via intra-articular route.

Results: Treatment of the chondrocytes with glucosamine cyclohexyl ester for 48 h prevented interleukin-1 β (IL-1 β)-mediated increases in mRNA and protein expressions in matrix metalloproteinases-1, -3 and -13, and also blocked IL-1 β -induced decreases in mRNA and protein expressions of tissue inhibitor of metalloproteinase-1. Glucosamine cyclohexyl ester treatment also blocked the onset of morphological changes such as irregular surface, adhesion of tissues and presence of osteophytes in the femoral condyle surface of the OA rats. Mankin score for control, OA and glucosamine cyclohexyl ester treatment groups were 0.98 ± 0.15 , 8.35 ± 0.88 and 2.39 ± 0.67 ($p = 0.002$), respectively. Treatment of OA rats with glucosamine cyclohexyl ester also inhibited increases in the activities of matrix metalloproteinases-1, -3 and -13, and decreases of tissue inhibitor of metalloproteinase-1 mRNA and protein expressions. Treatment of chondrocytes and OA rats with IL-1 β caused no significant changes in the levels of H3K27 and H4K8.

Conclusion: These results show that glucosamine cyclohexyl ester prevents OA by targeting the expressions of matrix metalloproteinases-1, -3 and -13 and tissue inhibitor of metalloproteinases-1.

Keywords: Metalloproteinases, Interleukin, Mankin score, Osteoarthritis, Cartilage

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INTRODUCTION

Osteoarthritis (OA) involves the failure of chondrocytes to function properly leading to degradation of extracellular matrix [1]. Chondrocytes are involved in the synthesis of extracellular matrix molecules for maintaining the

stability of the articular cartilage [2,3]. The function of chondrocytes is regulated by various molecules such as extracellular matrix and sulfated proteoglycans through interaction between the cells and matrix [4]. The development and progression of OA are characterized by joint inflammation [5].

Degradation of extracellular matrix molecules and inhibition of chondrocyte viability are due to the production of oxidants and IL-1 β in the activated synoviocytes and mononuclear cells [6,7]. Studies have revealed that IL-1 β induces expression of matrix metalloproteinases which inhibit the production of extracellular matrix molecules in chondrocytes [8,9]. Tissue inhibitors of metalloproteinase also enhance the activity of matrix metalloproteinases [10]. Disturbance in equilibrium between matrix metalloproteinases and tissue inhibitors of metalloproteinase leads to the development and progression of OA [10]. Patients with OA suffer continuously from pain and movement disability [11].

D-Glucosamine (GlcN) naturally present in connective and cartilage tissues prevents cartilage degradation by normalizing metabolism in cartilage and promoting the formation of proteoglycans [12]. It regulates the strength of cartilage by providing flexibility and elasticity to the tissues [12]. These properties have been exploited in the use of GlcN for the treatment of OA patients [13-15]. It has been reported that GlcN modifies various symptoms of OA in patients [16]. Indeed, the symptom-modifying and relieving effect of glucosamine in the knee of OA patients has also been recognized by Osteoarthritis Research Society International (OARSI) [17]. Studies have shown that GlcN treatment suppresses oxidants and IL-8 expression through its anti-oxidant effects [18].

In the present study, the role of glucosamine cyclohexyl ester (a GlcN derivative) in OA treatment in a rat model of OA was investigated.

EXPERIMENTAL

Reagents and chemicals

Glucosamine was obtained from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Dimethyl sulfoxide, interleukin-1 β , MTT and Dulbecco's modified Eagle's medium (DMEM) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Synthesis of glucosamine cyclohexyl ester

Glucosamine cyclohexyl ester was synthesized from glucosamine in a reaction with cyclohexyl iodide in presence of potassium carbonate, with tetrahydrofuran as solvent. The reaction mixture was refluxed at 80 °C till completion (1 h). The end of reaction was indicated by thin layer chromatography. The product was then purified by silica gel column chromatography and characterized using NMR studies.

Chondrocytes isolation and culture

The rats were acclimatized to the laboratory conditions for seven days before starting the study. Cartilage was excised from the knee and hip joints of the animals. The cartilage was digested for 5 h in DMEM with 0.2 % collagenase II (Sigma-Aldrich). The digest was filtered through filter mesh (pore size = 150 μ m) and subsequently subjected to centrifugation at 300 x g for 10 min at 4 °C to obtain the chondrocytes. The chondrocytes were cultured in DMEM medium supplemented with FBS (10 %) at a temperature of 37 °C. The culture medium also contained penicillin (100 U/mL) and streptomycin (100 μ g/mL).

Determination of effect of glucosamine cyclohexyl ether on chondrocyte viability

The chondrocytes were put into 96-well culture plates at a density of 5 x 10⁵ cells per well and left undisturbed overnight. The chondrocytes were then treated with 0.1, 0.2, 0.4, 0.8 and 1.6 μ M of glucosamine cyclohexyl ester for 48 h in DMEM medium devoid of serum. Following incubation of the chondrocytes, the medium was removed and 10 μ L of CCK-8 solution added to each well. The plates were then incubated for 4 h at 37 °C, and the absorbance of each well was read three times in a microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA) at 465 nm.

Analysis of matrix metalloproteinases and tissue inhibitor of metalloproteinases-1 in chondrocytes

Into a 6-well culture plate chondrocytes were put at a density of 2 x 10⁴ cells per well. After achieving 75 % confluence, the chondrocytes were subjected to serum deprivation for 24 h, and stimulated with interleukin-1 β at a concentration of 5 ng/mL for 12 h. The chondrocytes were then treated with glucosamine cyclohexyl ester at doses of 0.1, 0.2, 0.4, 0.8 and 1.6 μ M for 48 h, and cultured. Thereafter, the cells were collected for analysis of matrix metalloproteinase and tissue inhibitor of metalloproteinase-1 mRNA and protein expressions.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) was used for the determination of matrix metalloproteinase and tissue inhibitor of metalloproteinase-1 mRNA. The chondrocytes and cartilage tissues were

treated with TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) for the extraction of RNAs, which were reverse-transcribed to cDNA using prime Script-RT reagent kit (Takara Bio, Inc., Otsu, Japan). The cDNAs were amplified and quantified with qPCR (ABI Prism 7500; Applied Biosystems; Thermo Fisher Scientific, Inc.) and SYBR Premix Ex Taq (Takara Bio, Inc.).

The procedure for qPCR involved holding for 30 sec at 95 °C, cycling for 5 sec at 95 °C, followed by 30 sec at 70 °C. Forty cycles were performed. The primers for matrix metalloproteinase and tissue inhibitor of metalloproteinase-1 were obtained from Sangon Biotech Co., Ltd. (Shanghai, China). For matrix metalloproteinase-1, the forward and backward sequences were 5'-GGAACAGATACGAAGAGGAAACA-3' and 5'-TGTGGGAATCAGAGGTAGAAGA-3' respectively; for matrix metalloproteinases-3, the forward and backward sequences were 5'-GCATTGGCTGAGTGAAAGAGAC-3' and 5'-ATGATGAACGATGGACAGATGA-3' respectively; for matrix metalloproteinase-13, the sequences were 5'-TGAGAGTCATGCCAACAAATTC-3' and 5'-CAGCCACGCATAGTCATGTAGA-3'; for tissue inhibitor of metalloproteinase-1, the sequences were 5'-GGTTCCTGGCATAATCTGAG-3' and 5'-ATCGCTCTGGTAGCCCTTCT-3', respectively.

Western blot analysis

The protein expressions of matrix metalloproteinases and tissue inhibitor of metalloproteinase-1 in chondrocyte and cartilage tissues were analyzed using western blot assay. Proteins were extracted from the chondrocyte and cartilage tissues with lysis buffer (Wuhan Boster Biological Technology, Ltd., Wuhan, China) for 45 min., and quantified using bicinchoninic acid method (Thermo Fisher Scientific, Inc.). The protein samples were then subjected to sodium dodecyl sulfate-polyacrylamide gel (SDS) gel electrophoresis at 115 V for 2 h. The proteins were subsequently put onto polyvinylidene difluoride membranes. After incubation with primary antibodies overnight at a dilution of 1:1000, the membranes were washed and incubated for 1 h with horseradish peroxidase (HRP)-conjugated secondary goat anti-rabbit IgG antibody at a dilution of 1:5000 (Santa Cruz Biotechnology, Inc; catalog no., sc-2004) at room temperature. The resultant bands were examined using enhanced chemiluminescence (ECL; Thermo Fisher Scientific, Inc.). Quantity One version 4.6.2 software (Bio-Rad Laboratories Inc., Munich, Germany) was used for densitometry. The

primary antibodies used were matrix metalloproteinase-1 (catalog no., BS1229), matrix metalloproteinase-13 (catalog no., BS6668; both from Bioworld Technology, Inc.), matrix metalloproteinase-3 (catalog no., 17, 873-1-AP; ProteinTech Group, Inc., Chicago, IL, USA) and tissue inhibitor of metalloproteinase-1 (catalog no., sc-5538; Santa Cruz Biotechnology, Inc, Dallas, TX, USA).

Experimental animals

All experimental procedures were conducted according to the institutional guidelines and also followed the guidelines of National Research Council Guide for Care and Use of Laboratory Animals [19]. Fifteen (8 male and 7 female) Sprague-Dawley rats weighing 190 - 210 g were purchased from the Laboratory Animal Center, Guangzhou University of Traditional Chinese Medicine. The animals were maintained in animal center under humidity-controlled conditions at 25 °C. The rats were allowed access to standard laboratory chow and clean drinking water *ad libitum*. The animal experiments were performed with approval from the Animal Care and Use Committee of Guangzhou University of Traditional Chinese Medicine.

The rats were assigned randomly to three groups (5 per group): normal control, osteoarthritis (OA) and treatment group. The rats in OA and treatment groups were anesthetized using pentobarbital sodium (2 %) and subsequently injected with monoiodoacetate intra-articularly through the knee joint. Rats in the normal control group were given equal volume of normal saline. The treatment group of rats were injected 5 mg/kg of glucosamine cyclohexyl ester alternately for one month after one week of monoiodoacetate administration through intra-articular route. The rats were sacrificed by cervical dislocation under pentobarbital sodium (2 %) anesthesia.

Histopathological examination

Cartilage samples were excised and treated for 70 h with paraformaldehyde. The samples were subsequently decalcified for 6 h by exposure to methanoic acid. The decalcified tissues were embedded in paraffin and then sliced into 4 mm sections. After heating in boiling xylene the sections were treated with Safranin-O stain. Mankin score was calculated for the tissue sections in accordance with standard protocol [20].

Statistical analysis

Data are presented as mean \pm standard deviation. Student's *t*-test was used for the comparison of data among various groups. Moreover, one-way analysis of variance (ANOVA) and Dunnett's analysis were used. Differences were considered statistically significant at $p < 0.05$.

RESULTS

Effect of glucosamine cyclohexyl ester on chondrocyte viability

Glucosamine cyclohexyl ester exhibited no significant inhibitory effect on the viability of chondrocytes up to 0.8 μ M (Figure 1).

Effect of glucosamine cyclohexyl ester treatment on mRNAs of metalloproteinases

The levels of mRNA expressions of matrix metalloproteinases-1, -3 and -13 in chondrocytes were increased when the chondrocytes were cultured in presence of IL-1 β . Culturing of chondrocytes in presence of IL-1 β led to a significant decrease in the expression level of tissue inhibitor of metalloproteinase-1 (Figure 2). However, treatment of the chondrocytes with glucosamine cyclohexyl ester for 48 h reversed the effect of IL-1 β on the expression of matrix metalloproteinases and tissue inhibitor of metalloproteinase-1 expression. Results from western blot analysis were also consistent with those from RT-PCR assay. Glucosamine cyclohexyl ester treatment reversed the IL-1 β -induced increases in the protein expressions of matrix metalloproteinases-1, -3 and -13. Similarly, the IL-1 β -induced decrease in the tissue inhibitor of metalloproteinase-1 protein

expression was reversed by glucosamine cyclohexyl ester treatment (Figure 2). Glucosamine cyclohexyl ester treatment of chondrocytes without pretreatment with IL-1 β promoted the protein expression of tissue inhibitor of metalloproteinases-1 without influencing the expressions of matrix metalloproteinases-1, -3 and -13 protein (Figure 2).

Glucosamine cyclohexyl ester prevents knee joint degeneration in OA rat model

Examination of femoral condyles showed marked changes in cartilage morphology such as irregular surface, adhesion of tissues and presence of osteophytes in the OA rats. However, in the control group of rats, the femoral condyle surface was normal. Femoral condyle surface in the OA rats treated with glucosamine cyclohexyl ester also showed no morphological changes. The protective effect of glucosamine cyclohexyl ester against deleterious changes in surface morphology in OA rats was also confirmed using Safranin-O staining (Figure 3). The cartilage of rats in the control group showed markedly degree of Safranin-O staining when compared to the OA rats. Prevention of cartilage degradation by glucosamine cyclohexyl ester treatment in the OA rats was evident in the higher level of Safranin-O staining in the treated OA rats relative to the OA rats (Figure 3).

Analysis of cartilage histology revealed a markedly higher Mankin score in the OA rat group when compared to the normal control rats. The Mankin scores in control, OA and glucosamine cyclohexyl ester treatment groups of rats were 0.98 ± 0.15 , 8.35 ± 0.88 and 2.39 ± 0.67 , respectively ($p < 0.002$).

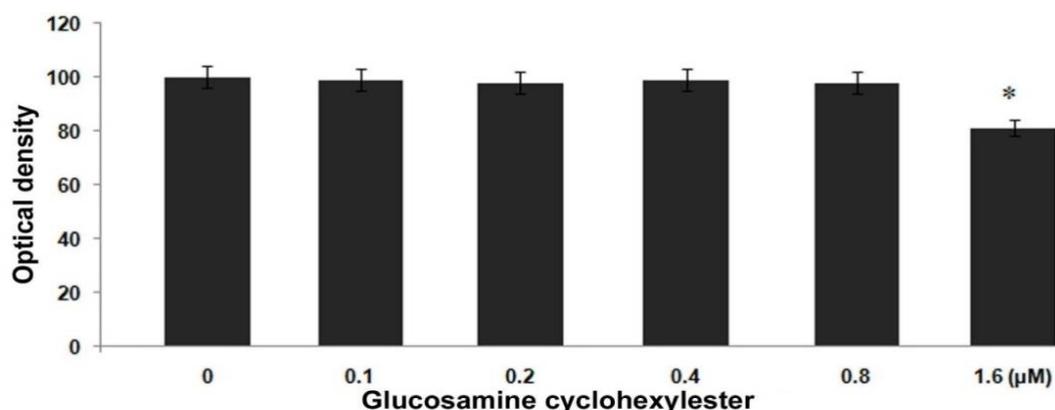


Figure 1: Determination of optimal dose of glucosamine cyclohexyl ester using CCK-8 assay. The chondrocytes were cultured with 0.1, 0.2, 0.4, 0.8 and 1.6 μ M of glucosamine cyclohexyl ester for 48 h. For each of the doses, viability measurement was performed in triplicates and data are expressed as mean \pm S.D. * $p < 0.01$ compared to control without glucosamine cyclohexyl ester

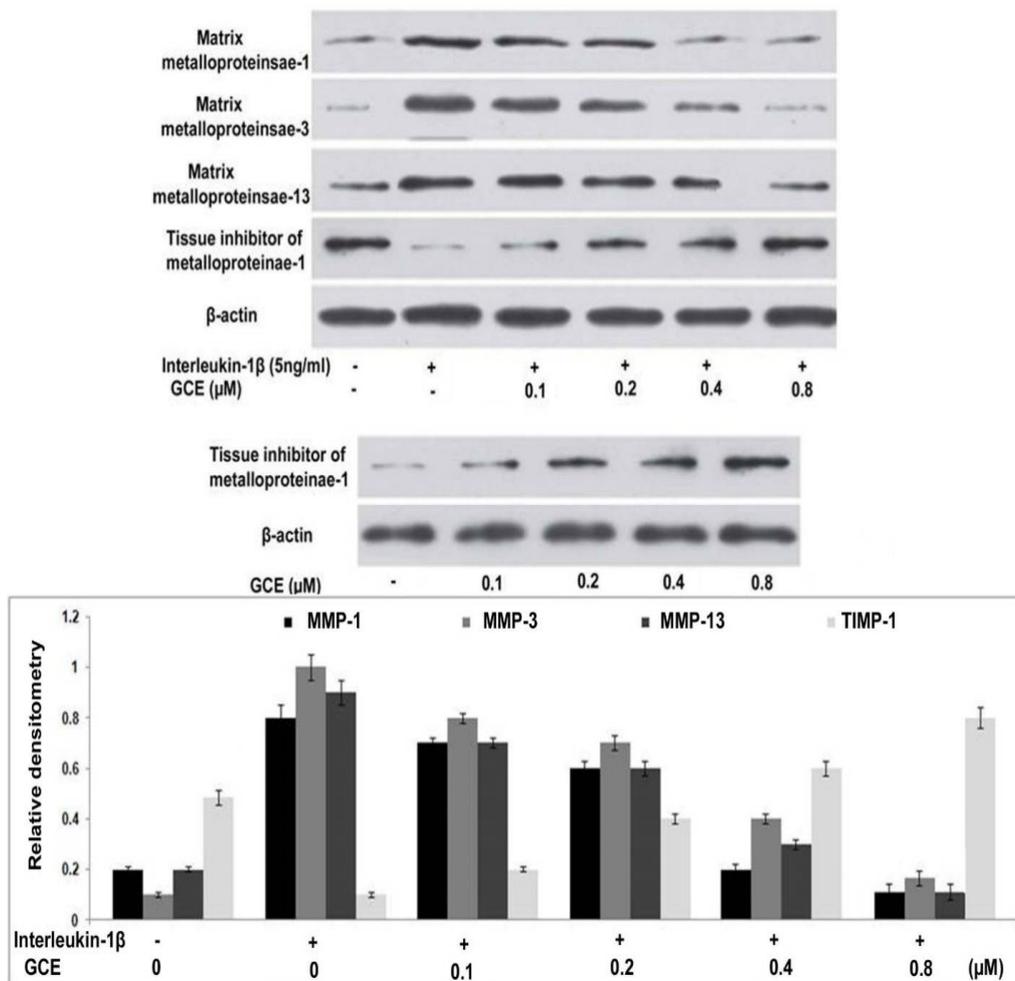


Figure 2: Effect of glucosamine cyclohexyl ester on levels of matrix metalloproteinases-1, -3, and -13; and tissue inhibitor of metalloproteinase-1 expressions in chondrocytes. Data are presented as mean ± SD. **p* < 0.02 compared to chondrocytes without glucosamine cyclohexyl ester treatment.

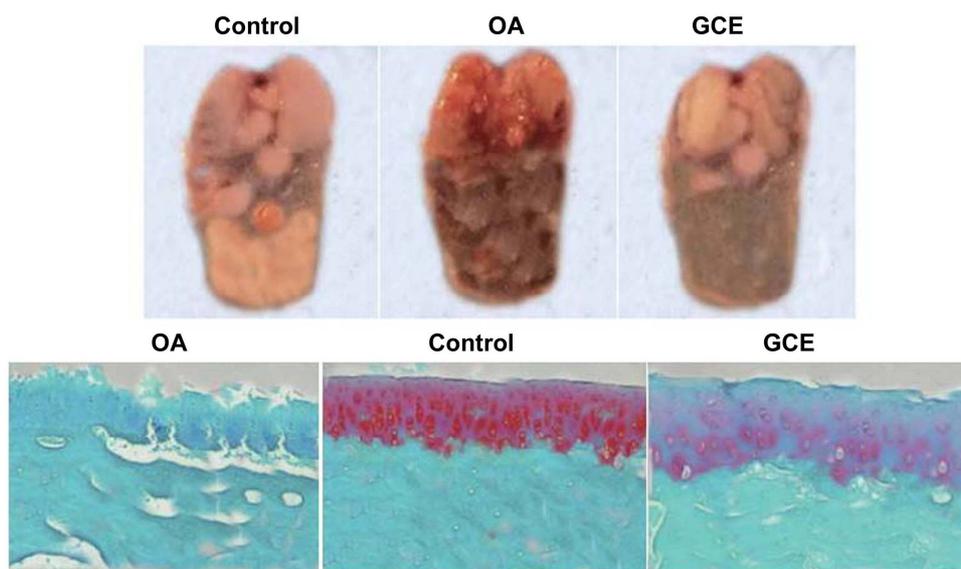


Figure 3: Effect of glucosamine cyclohexyl ester on cartilage degradation in OA rats. Cartilage degradation after glucosamine cyclohexyl ester treatment was examined using Safranin-O stain. The images were captured at a magnification of x100

Glucosamine cyclohexyl ester affects expressions of matrix metalloproteinases-1, -3 and -13, and tissue inhibitor of metalloproteinase-1 *in vivo*.

Examination of cartilage using RT-qPCR analysis showed higher expression of matrix metalloproteinases-1, -3 and -13 mRNA in OA rats, when compared to control group. The level of tissue inhibitor of metalloproteinases-1 mRNA in OA rats was significantly reduced relative to the control group ($p < 0.05$; Figure 4). However, treatment of OA rats with glucosamine cyclohexyl ester significantly reduced the increases in matrix metalloproteinases-1, -3 and -13 mRNA expression, and decreased levels of tissue inhibitor of metalloproteinases-1 mRNA ($p < 0.05$). These findings from RT-qPCR analysis were consistent with results from western blot analysis (Figure 4).

Glucosamine cyclohexyl ester increases acetylation levels in chondrocytes and cartilage

Glucosamine cyclohexyl ester increased the acetylation of H3K27 and H4K8 in chondrocytes *in vitro* and in cartilage of OA rats *in vivo* (Figure 5).

DISCUSSION

The present study was performed with the aim of investigating the role of glucosamine cyclohexyl ester in osteoarthritis treatment. Glucosamine cyclohexyl ester increased chondrocyte viability and prevented cartilage degradation in the OA rat model through targeting the expression levels of mRNAs and protein in matrix metalloproteinases-1, -3 and -13, and tissue inhibitor of metalloproteinase-1. Tissue inhibitor of metalloproteinases-1 plays an important role in the degeneration of cartilage by promoting the expression of matrix metalloproteinases [21]. Increases in the level of matrix metalloproteinases and down-regulation of tissue inhibitor of metalloproteinases-1 promote cartilage degradation and inhibit chondrocyte viability [21].

It has been reported that the degradation of several components of extracellular matrix such as type II collagen, is catalyzed by matrix metalloproteinases [22]. Degradation of type II collagen by matrix metalloproteinases is a characteristic feature of OA [22].

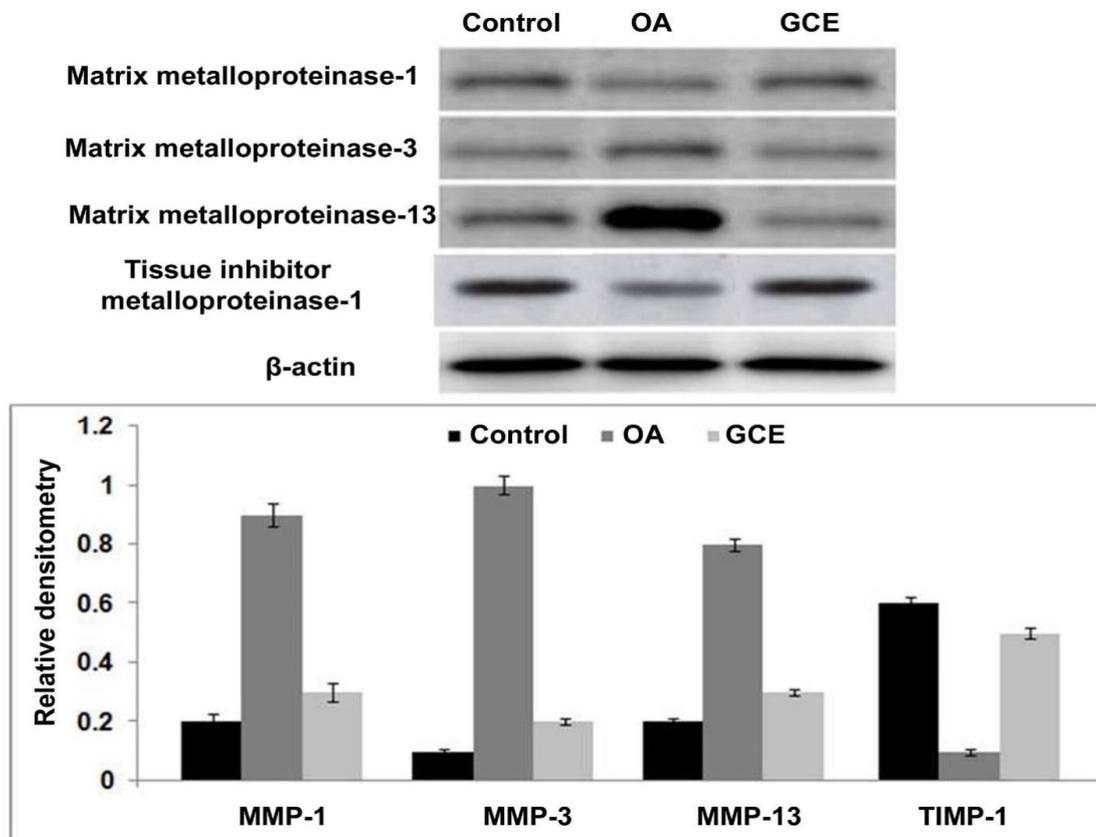


Figure 4: Effect of glucosamine cyclohexyl ester on expressions of matrix metalloproteinases-1, -3, and -13; and tissue inhibitor of metalloproteinase-1 in OA rats

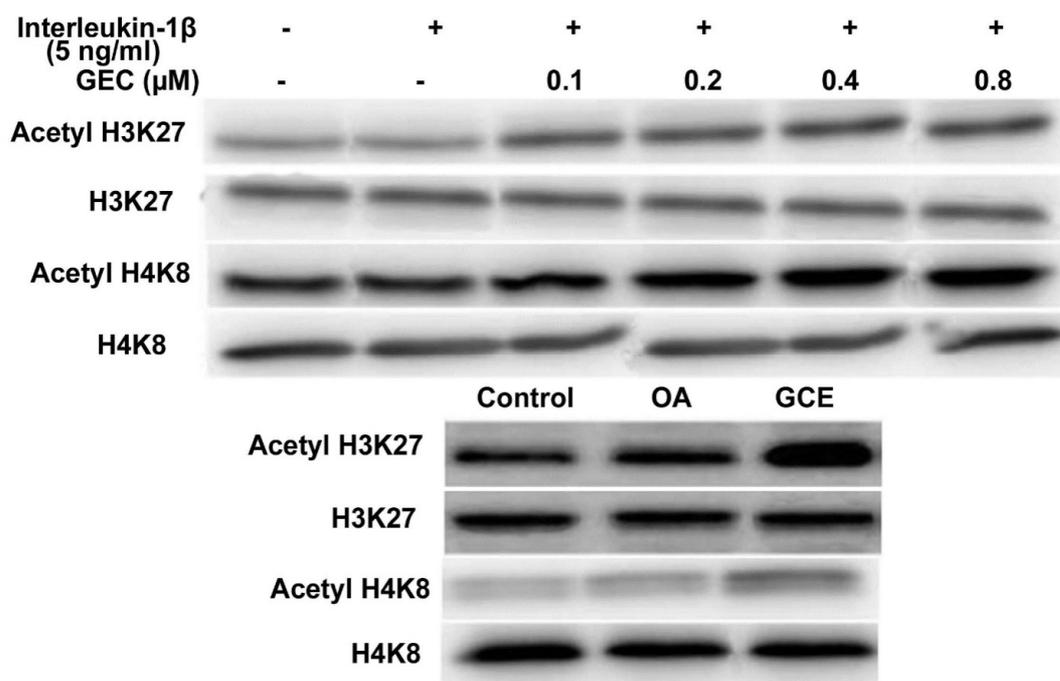


Figure 5: Effect of glucosamine cyclohexyl ester on acetylation of H3K27 and H4K8. The rats were treated with glucosamine cyclohexyl ester for 48 h after 12 h of pretreatment with IL-1 β and then H3K27 and H4K8 acetylation was analyzed using western blot assay

In the current study, glucosamine cyclohexyl ester significantly inhibited the expression of matrix metalloproteinases-1, -3 and -13 mRNA and protein in the chondrocytes of OA rats, when compared to untreated OA rats. Glucosamine cyclohexyl ester also blocked the IL-1 β mediated decrease in the levels of tissue inhibitor of metalloproteinases-1 mRNA and protein *in vitro*.

Western blot and RT-PCR assay results showed markedly higher levels of matrix metalloproteinases and lower level of tissue inhibitor of metalloproteinases-1 mRNA and protein in the OA rats *in vivo*. However, treatment of the OA rats with glucosamine cyclohexyl ester prevented the increase in matrix metalloproteinases-1, -3 and -13 expressions, and decreased tissue inhibitor of metalloproteinases-1 mRNA and protein. Examination of femoral condyles of the OA rats showed marked changes in morphology such as irregular surface, adhesion of tissues and presence of osteophytes, while the femoral condyle surface in the OA rats treated with glucosamine cyclohexyl ester showed no morphological changes. This is an indication of the protective effect of glucosamine cyclohexyl ester against OA. This protective effect was confirmed by results from Safranin-O staining, which showed that glucosamine cyclohexyl ester treatment markedly reduced the Mankin score in OA rats, when compared to untreated OA rats.

CONCLUSION

The findings of this study demonstrate that glucosamine cyclohexyl ester has immense therapeutic potential in the management of OA.

DECLARATIONS

Acknowledgement

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Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them.

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