Chondroprotective activity of \(N\)-acetylglucosamine in rabbits with experimental osteoarthritis

A R Shikhman, D Amiel, D D’Lima, S-B Hwang, C Hu, A Xu, S Hashimoto, K Kobayashi, T Sasho, M K Lotz

Objective: To examine the therapeutic efficacy of \(N\)-acetylglucosamine (GlcNAc) in rabbits with experimental osteoarthritis (OA).

Methods: Experimental OA was induced in rabbits by anterior cruciate ligament transection (ACLT). In the first study, rabbits (six in each group) received intramuscular injections of GlcNAc or normal saline three times a week starting 1 week postoperatively. In the second study, rabbits (eight in each group) were injected intra-articularly with GlcNAc (either once or twice a week) or normal saline. In the third study, rabbits (seven in each group) were injected intra-articularly twice a week with either GlcNAc, hyaluronan, or normal saline. Animals were killed 8 weeks after ACLT for macroscopic and histological assessment of the knee joints.

Results: Intramuscular administration of GlcNAc in rabbits with experimental knee OA did not show chondroprotective effects but showed mild anti-inflammatory activity. In contrast, intra-articular administration of GlcNAc twice a week reduced cartilage degradation. Additionally, intra-articular GlcNAc also suppressed synovitis. Once a week intra-articular injections of GlcNAc did not demonstrate therapeutic efficacy. The chondroprotective efficacy of GlcNAc was better than that of viscosupplementation treatment with hyaluronan.

Conclusion: Intra-articular GlcNAc has chondroprotective and anti-inflammatory activity in experimental OA.

Osteoarthritis (OA) is the most common joint disease, which is characterised by progressive cartilage degeneration, subchondral bone changes, and chronic synovitis. Current treatment of OA is limited to symptom management with analgesics, anti-inflammatory agents, and viscosupplementation devices. Glucosamine (GlcN) is widely used by patients with OA and may modify disease activity. It has been suggested that GlcN delays progressive joint space narrowing and improves the biomechanics of OA knee joints even in the absence of pain relief.

Mechanisms of GlcN mediated anti-arthritic activities remain to be defined. A limitation of in vitro studies on GlcN is that anti-inflammatory and chondroprotective GlcN effects are predominantly observed at lower millimolar concentrations, which exceed the range of therapeutic concentrations obtained with oral GlcN by a factor of \(10^{3} - 10^{4}\). Although maximal anti-arthritic efficacy of GlcN and its derivatives can be expected upon intra-articular administration of the sugars at high concentrations, detailed analyses of local use of these agents have not been reported.

In this study \(N\)-acetylglucosamine (GlcNAc) was selected as the preferred agent for intra-articular administration. This was based on our previous observations that GlcNAc was more efficient than GlcN in inhibiting interleukin \(\beta\) mediated activation of human articular chondrocytes in vitro. In addition, GlcNAc at concentrations exceeding 10 mmol/l significantly decreased chondrocyte viability in vitro, which potentially complicates its intra-articular administration. In contrast, GlcNAc did not induce chondrocyte cell death even at concentrations above 50 mmol/l.

This study examined the efficacy of intramuscularly administered GlcNAc. The second study determined the anti-arthritic activity of intra-articular GlcNAc and the best frequency for the injections. The third study compared the efficacy of intra-articular GlcNAc with that of intra-articular hyaluronan, which is a viscosupplementation device currently used in patients with knee OA.

MATERIALS AND METHODS

Reagents
GlcNAc was purchased from Sigma (St Louis, MO). It was dissolved in normal saline and sterilised by filtration through 0.22 \(\mu\)m filters (Corning, Acton, MA). Sterile solutions of GlcNAc were stored at 4°C. Sodium hyaluronate (Hyalgan) was purchased from Sanofi-Synthelabo (New York, NY).

Animals
New Zealand White rabbits, age 8–12 months, weight 3.7–4.2 kg, and with closed epiphyses, were used in all experiments. All studies were performed in accordance with The American Association of Laboratory Animal Science guidelines and after approval of The Scripps Research Institute and the University of California, San Diego animal review committees. A total of 57 rabbits were used in the experiments.

ACLT
Unilateral or bilateral ACLT was performed as indicated for each set of experiments using a medial arthrotomy technique. All animals were maintained individually and were

Abbreviations: ACLT, anterior cruciate ligament transection; GAG, glycosaminoglycan; GlcN, glucosamine; GlcNAc, \(N\)-acetylglucosamine; OA, osteoarthritis
freely active. The animals were killed 8 weeks after the surgery. Previously published data showed that most rabbits with ACLT develop cartilage degeneration at this time.20 21 Intramuscular injections of GlcNAc Intramuscular injections of GlcNAc were performed three times a week starting 1 week postoperatively for a period of 7 weeks. The dose of GlcNAc was 200 mg/kg for each injection. The control group received the same number of intramuscular injections of normal saline.

Intra-articular injections of GlcNAc and hyaluronan Intra-articular injections of GlcNAc started 1 week postoperatively for a period of 7 weeks. Rabbits were injected once or twice a week with GlcNAc in a volume of 0.3 ml for each knee joint. The single dose of GlcNAc for each injection was 80 mg. Control animals were injected twice a week intra-articularly with normal saline (0.3 ml per joint). Intra-articular injections of hyaluronan (3 mg/0.3 ml per joint) started 1 week postoperatively for a period of 7 weeks. Synovial fluid analysis was performed in three animals who developed gross synovial effusions (two animals in the control group and one in the hyaluronan group). In all three animals the synovial fluid was culture negative.

Gross morphological assessment of the knee joints

Gross morphological assessment of the knee joints was performed in a blinded fashion and included evaluation of tibial plateaus and femoral condyles. The distal femur and proximal tibia were harvested, keeping the 3.5–4 cm shaft of the bones. The articular cartilage surface of each specimen was covered with a solution consisting of India ink (Eberhard Faber, Lewisburg, TN) in phosphate buffered saline (1:5 ratio). Excess ink solution was removed by gentle blotting with a tissue that was pre-moistened with phosphate buffered saline. Subsequently, all joints were photographed and digital images were analysed.

The grading system for articular cartilage assessment was as follows:

- Grade 1 (intact surface)—surface is normal in appearance and does not retain India ink

Figure 1. Morphological grading of femoral condyles (A) and tibial plateaus (B) in rabbits with unilateral ACLT treated with intra-articular GlcNAc or saline. A total of 24 rabbits underwent unilateral ACLT. Eight rabbits were injected intra-articularly with GlcNAc once a week in a volume of 0.3 ml per knee joint. The single dose of GlcNAc for each injection was 80 mg. The second group of rabbits received intra-articular injections of GlcNAc twice a week in a volume of 0.3 ml per knee joint (80 mg for each injection). A control group of seven animals was injected intra-articularly twice a week with normal saline (0.3 ml per joint). All intra-articular injections started 1 week postoperatively and were performed for a period of 7 weeks.

Figure 2. Gross morphological grading of femoral condyles (A) and tibial plateaus (B) in rabbits with unilateral ACLT treated with intra-articular GlcNAc, hyaluronan, or saline. A total of 21 rabbits underwent unilateral ACLT. Seven rabbits were injected twice a week intra-articularly with GlcNAc in a volume of 0.3 ml per knee joint. The single dose of GlcNAc for each injection was 80 mg. The second group of rabbits received twice a week intra-articular injections of hyaluronan (3 mg/0.3 ml per joint). The control group of seven animals was injected twice a week intra-articularly with normal saline (0.3 ml per joint). All intra-articular injections started 1 week postoperatively and were performed twice a week for a period of 7 weeks.
• Grade 2 (minimal fibrillation)—surface retains India ink as elongated specks or light grey patches
• Grade 3 (overt fibrillation)—areas which are velvety in appearance and retain India ink as intense black patches
• Grade 4 (erosion)—loss of cartilage exposing the underlying bone.

Digital imaging
Articular surfaces of femoral condyles and tibial plateaus were gently blotted dry and cleaned of loose tissue. Each femoral shaft was clamped to an optical bench. An image (resolution: 60 pixels/mm; onscreen magnification: 20 x) of the femoral condyles was obtained using a Canon EOS D30 digital camera with a 100 mm macro lens at a distance of approximately 12 cm. A millimetre scale was included in the photograph to scale the image accurately. The scaled image was then projected onto a three dimensional model of the femoral condyles. The three dimensional surface area of the lesion was measured by interactively plotting the margins of the lesion. A digital image of the articular surface of the tibia was obtained as described above. No three dimensional projection was used because the tibial surface was relatively flat and two dimensional measurements do not vary significantly from three dimensional measurements.

Histological grading of the knee joints
Distal femur and proximal tibia from the rabbit knee joints were fixed in 10% buffered formalin, decalcified in TBD-2 decalcifier (ThermoShandon, Pittsburg, CA) and embedded in paraffin blocks. Sagittal sections of lateral and medial femoral condyles, and coronal sections of tibial plateaus were used for histological analysis.

The assessment of sulphated glycosaminoglycan (GAG) content was performed after staining of the tissue sections with safranin O/ fast green.

The grading system for assessment of sulphated GAG was as follows:
• Grade 1—less than 25% loss of safranin O staining
• Grade 2—25–50% loss of safranin O staining
• Grade 3—more than 50% loss of safranin O staining.

The grading system for assessment of cartilage integrity was as follows:
• Grade 1—intact cartilage surface
• Grade 2—presence of fibrillations
• Grade 3—full thickness cartilage defect.

Histological assessment of synovitis was based on the presence of synovial proliferation and villous structures, and it was performed separately for the synovium attached to the tibial plateaus, lateral and medial femoral condyles. Monolayer synovial lining without villous structures was considered to be normal (grade 0). Hypertrophic synovium and synovium with villous elements were considered to be abnormal (grade 1).

Statistical analysis
Statistical analysis of the experimental data was performed using Fisher’s exact test and two tail t test: two samples paired for means (for digital data analysis).

RESULTS
Intramuscular GlcNAc in rabbits with experimental OA
The anti-arthritic activity of GlcNAc was assessed in three independent sets of experiments. The first series of experiments analysed the activity of high dose GlcNAc administered through intramuscular injections. The efficacy of intramuscular GlcNAc was assessed in six rabbits with bilateral ACLT and compared with a control group of six rabbits, who also underwent bilateral ACLT and received intramuscular saline injections. Gross morphological analysis of tibial plateaus and femoral condyles did not show significant differences in the degree of cartilage damage between GlcNAc treated and control animals. Histological examination of the knee joints did not demonstrate statistically significant differences in cartilage sulphated glycosaminoglycan (GAG) content.

Table 1 Histological grading of articular cartilage

<table>
<thead>
<tr>
<th>Anatomical area</th>
<th>Saline (n = 7)</th>
<th>Intra-articular GlcNAc (n = 6)</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sulphated glycosaminoglycan loss</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Medial femoral condyle</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;25%</td>
<td>3/7</td>
<td>3/6</td>
<td>0.77</td>
</tr>
<tr>
<td>25–50%</td>
<td>4/7</td>
<td>2/6</td>
<td></td>
</tr>
<tr>
<td>50–75%</td>
<td>0/7</td>
<td>1/6</td>
<td></td>
</tr>
<tr>
<td>Lateral femoral condyle</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;25%</td>
<td>0/7</td>
<td>1/6</td>
<td>0.56</td>
</tr>
<tr>
<td>25–50%</td>
<td>4/7</td>
<td>1/6</td>
<td></td>
</tr>
<tr>
<td>50–75%</td>
<td>3/7</td>
<td>1/6</td>
<td></td>
</tr>
<tr>
<td>Tibial plateau</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;25%</td>
<td>3/7</td>
<td>5/6</td>
<td>0.40</td>
</tr>
<tr>
<td>25–50%</td>
<td>3/7</td>
<td>1/6</td>
<td></td>
</tr>
<tr>
<td>50–75%</td>
<td>1/7</td>
<td>0/6</td>
<td></td>
</tr>
</tbody>
</table>

Table 2 Histological grading of synovium

<table>
<thead>
<tr>
<th>Anatomical area</th>
<th>Saline (n = 7)</th>
<th>Intra-articular GlcNAc (n = 6)</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Synovial thickening/proliferation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Medial femoral condyle</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7/7</td>
<td>2/6</td>
<td>&lt;0.021*</td>
<td></td>
</tr>
<tr>
<td>Lateral femoral condyle</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7/7</td>
<td>2/6</td>
<td>&lt;0.021*</td>
<td></td>
</tr>
<tr>
<td>Tibial plateau</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7/7</td>
<td>1/6</td>
<td>&lt;0.0047*</td>
<td></td>
</tr>
</tbody>
</table>

Synovial membranes from GlcNAc and saline treated rabbits (third study) were microscopically analysed for the presence of synovitis as described in “Materials and methods”.

*Indicates significant differences.

Figure 3 Digital image analysis of the lesion size in femoral condyles (A) and tibial plateaus (B). Digital image analysis of the lesion size was performed in seven animals with unilateral ACLT treated with intra-articular GlcNAc and in seven animals with unilateral ACLT treated with intra-articular saline.
Anti-arthritic activity of intra-articular GlcNAc

As maximal biological activity of GlcNAc in vitro was seen at low millimolar concentrations,25 we examined the efficacy of high dose intra-articular GlcNAc treatment in experimental OA. The experiments included three groups of rabbits. Rabbits in the first group were treated with intra-articular GlcNAc injections twice a week; rabbits in the second group were treated with intra-articular GlcNAc injections once a week; and the third group of rabbits received intra-articular normal saline. The duration of the treatment was 7 weeks.

Gross morphological analysis of the femoral condyles showed that animals treated twice a week with intra-articular GlcNAc had a trend towards reduced severity of cartilage lesions as compared with the control group (p<0.11); once a week intra-articular administration of GlcNAc showed no significant benefits (fig 1A). Similar results were obtained in the analysis of the tibial plateaus: GlcNAc injections twice a week were associated with a beneficial therapeutic trend, p=0.18 (fig 1B). Statistical significance was not achieved, probably owing to the small size of the groups.

Results of macroscopic grading were confirmed by digital image analysis of cartilage lesions. The mean (SD) cartilage lesion area in control femoral condyles was 14.34 (12.86) mm$^2$ v 4.40 (7.57) mm$^2$ in the rabbits treated twice a week (p<0.02). Once a week GlcNAc administration did not significantly reduce the cartilage lesion area (13.74 (12.71) mm$^2$, p<0.88).

In general, cartilage lesions on the tibial plateaus were smaller than those on femoral condyles, probably reflecting a protective role of the menisci in tibial cartilage degeneration.26 Although the rabbits treated twice a week with GlcNAc had lesions 50% smaller than those in the control group (3.76 (6.69) mm$^2$ v 7.90 (8.44) mm$^2$), owing to the limited number of experimental animals, the differences between the groups were not significant (p<0.29). The rabbits treated once a week with GlcNAc did not demonstrate significant differences as compared with the control group (the mean cartilage lesion area was 9.59 (6.31) mm$^2$, p=0.48).

In conclusion, twice a week intra-articular administration of GlcNAc was better than once a week intra-articular GlcNAc and normal saline injections in reducing the severity of cartilage degeneration in the ACLT model of OA.

Therapeutic efficacy of intra-articular GlcNAc in comparison with intra-articular hyaluronan

The objectives of the third study were to confirm independently the chondroprotective efficacy of the intra-articular GlcNAc injections given twice a week and to compare the efficacy of intra-articular GlcNAc injections with intra-articular hyaluronan injections, which are used as visco-supplementation treatment in patients with knee OA.27–29 For this purpose, rabbits with unilateral ACLT were intra-articularly injected twice a week for 7 weeks with GlcNAc (seven rabbits), or hyaluronan (seven rabbits), or normal saline (seven rabbits).

Gross morphological analysis of the femoral condyles demonstrated a trend towards improvement of cartilage lesions in GlcNAc treated animals as compared with the control and hyaluronan treated groups, p<0.10 (fig 2A).

Examination of tibial plateaus disclosed the remarkable chondroprotective activity of GlcNAc as only 1/7 rabbits developed cartilage lesions, whereas 6/7 animals in the saline-injected group and 5/7 hyaluronan treated rabbits showed cartilage lesions (fig 2B). The chondroprotective effect of GlcNAc was statistically better than that of the saline group (p<0.013) and of the hyaluronan treated group (p<0.034).

Digital image analysis of femoral condyles (fig 3A) and tibial plateaus (fig 3B) from GlcNAc treated animals compared with hyaluronan treated animals demonstrated a trend towards greater efficacy of GlcNAc compared with hyaluronan.

Taken together, results of the third series of experiments confirmed the therapeutic efficacy of the GlcNAc injections administered twice a week and showed their superior chondroprotective activity in comparison with intra-articular hyaluronan treatment.

Histological analysis

Knee joints from seven control rabbits and six GlcNAc treated rabbits (from the third study) were used for histological analysis. One rabbit from the GlcNAc treated group was excluded from the study owing to identification of inclusion bodies in the articular cartilage.

Loss of sulphated GAG from cartilage at the medial femoral condyles and tibial plateaus was similar in GlcNAc treated and control rabbits (table 1). The lateral femoral condyles of GlcNAc treated rabbits showed a trend towards reduced sulphated GAG loss as compared with control animals (p=0.056). Examination of cartilage integrity demonstrated a trend towards chondroprotective activity in the tibial plateaus, p<0.10 (table 1).

GlcNAc was statistically better than normal saline in suppressing synovitis (table 2).

Collectively, intra-articular GlcNAc administration showed histological evidence of chondroprotective and anti-inflammatory activities.

DISCUSSION

Current pharmacotherapy of OA is limited to pain relief and improvement of viscoelastic properties of synovial fluid, but these interventions do not possess structure modifying activities. Recent results from multicentre clinical trials suggest that oral GlcN can reduce cartilage loss in OA joints.4 Despite the widespread use of GlcN by patients with OA, only limited data about its activity in experimental OA are available. In rabbits with experimental OA oral administration of GlcN slightly improved the morphological grade of OA lesions.26 In experimental arthritides induced by intra-articular chymopapain injections, oral GlcN preserved cartilage sulphated GAG content but significantly worsened the histopathological changes.27 Mechanisms that account for GlcN effects on OA joints are unclear. Its optimal in vitro activities are observed at millimolar concentrations which cannot be achieved with oral administration.22–16 Such concentrations can be obtained with intra-articular injections. Based on our previous observations, which showed that the in vitro anti-inflammatory activity and chondrocyte toxicity of GlcNAc were better than those of GlcN,31–18 the present study examined anti-arthritic activity of high dose GlcNAc in vivo after intramuscular versus intra-articular administration in rabbits with experimental OA.

Experimental OA in rabbits was induced by ACLT. This is one of the most widely used models to examine disease modifying treatments of OA.28 ACLT results in abnormal knee biomechanics, including increased anterior drawer at extension and at 90° of flexion, as well as an increased internal rotation similar to that seen in human OA knees.29 The most
severe areas of cartilage degeneration in rabbits with ACLT occur in medial femoral condyles followed by lateral femoral condyles. In the tibial plateau ACLT causes mild to moderate OA lesions in the areas not covered by the menisci.

Intramuscular administration of GlcNAc in rabbits with experimental OA did not demonstrate chondroprotective effects but showed a trend towards reduced synovitis. In two independent studies, twice a week intra-articular administration of GlcNAc showed a reduction in cartilage degradation. Once a week intra-articular GlcNAc injections failed to demonstrate therapeutic efficacy.

Inflammatory changes associated with the ACLT model include joint effusions and synovial membrane hyperplasia, which represent an important mechanism of joint pain in patients with OA. In the present study we observed anti-inflammatory activities of intra-articular GlcNAc with a reduction in the severity of synovitis. The observed antiarthritic effects of GlcNAc may, in part, be the consequence of its ability to suppress inflammatory responses. The direct effects of GlcNAc on chondrocytes include an inhibition of interleukin 1 responses, such as inhibition of expression of inducible nitric oxide synthase and cyclo-oxygenase. This may protect against chondrocyte death and cartilage matrix degradation. GlcNAc also inhibits inflammatory reactions in rats undergoing chronic peritoneal dialysis. Furthermore, orally administered GlcNAc was successfully used to control inflammation in children with Crohn’s disease and ulcerative colitis.

Among other GlcNAc properties contributing to its antiarthritic activity is a stimulatory effect on hyaluronic acid synthesis in human articular chondrocytes and synovial fibroblasts. Increased production of hyaluronic acid upon exposure to GlcNAc has also been seen in human peritoneal mesothelial cells and fibroblasts. Chronic peritoneal dialysis with a GlcNAc supplemented solution causes accumulation of GAG in the peritoneal interstitium and decreased peritoneal permeability.

Our study included a comparison of the anti-arthritic efficacy of GlcNAc with hyaluronic acid. Results from experimental OA and limited arthroscopic observations in humans indicate that hyaluronic acid possesses mild chondroprotective and anti-inflammatory activities. Compared with hyaluronic acid, intra-articularly administered GlcNAc in normal saline has a much shorter half life (minutes for GlcNAc versus hours for hyaluronic acid (San-Bao Hwang, unpublished data)). However, in experimental OA GlcNAc demonstrated better disease modifying activity than hyaluronic acid. This observation opens an avenue for design of GlcNAc formulations with optimised pharmacokinetic parameters to obtain even greater therapeutic efficacy in OA.

Adverse reactions with systemically or locally administered GlcNAc were not seen in this study. Published data also indicate that GlcNAc has an excellent safety profile in humans. Intravenous infusion of up to 100 g/h of GlcNAc in adult healthy volunteers was well tolerated. No significant changes in serum glucose and ammonia concentrations were noticed. Oral or rectal administration of GlcNAc in children with inflammatory bowel disease also did not produce adverse reactions.

In summary, this study identifies GlcNAc as the first N-acetylated amino sugar with disease modifying and anti-inflammatory activities in experimental OA.

ACKNOWLEDGEMENTS

We thank Ilo Creighton-Achermann for excellent technical help with the histological analysis and Dr James A Koziol for help with statistical analysis of the data. This work was supported by the National Institutes of Health grants AG07996, AT00052, and AR46990.


**ECHO**

*Popularity uncovers COX-2 inhibitor side effects*

An ecological study seems to suggest that cyclo-oxygenase (COX)-2 inhibitors result in adverse drug reactions at population level, even though they are judged safer than previous non-steroidal anti-inflammatory drugs (NSAIDs) for individual patients. Doctors in Canada performed a cross sectional time series study with data from administrative healthcare databases covering 1.3 million Ontario residents aged 66 years and over and compared the prevalences of NSAIDs use and upper gastrointestinal bleeding needing hospital admission.

A 41% increase in use of COX-2 inhibitors during 1994–2002 was mirrored by a 10% increase in hospital admission rates. Plotting the prevalences over time showed a parallel rise after COX-2 inhibitors were introduced in April 2000 and March 2001. NSAIDs use after COX-2 inhibitors were introduced rose by a prevalence of 14% to 19.8%, representing 650 extra bleeds. The researchers are confident that the time trends indicate a strong direct link, and they found no evidence for other explanations. What they cannot say, though, is whether increased pain relief afforded by COX-2 inhibitors offset the disbenefits of the side effects.

COX-2 inhibitors carry less risk of gastrointestinal bleeding than non-selective NSAIDs, according to recent evidence, but it was uncertain whether this was true with the wider use of these newer drugs and greater exposure at the population level.

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Ann Rheum Dis 2005 64: 89-94
doi: 10.1136/ard.2003.019406