

Positive Effect of Oral Supplementation with Glycosaminoglycans and Antioxidants on the Regeneration of Osteochondral Defects in the Knee Joint

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Summary

The effect of oral supplementation with glycosaminoglycans (GAG) and radical scavengers (vitamin E/selenium) on the regeneration of osteochondral defects was investigated in rabbits. After introduction of defined osteochondral defects in the knee joint, groups of ten animals were given a GAG/vitamin E/selenium mixture or a placebo (milk sugar) for 6 weeks. Following sacrifice, histological and histochemical analysis was performed. The amount of synovial fluid was increased in the placebo group, while the viscosity of the synovial fluid was significantly enhanced in the GAG group. The amount of sulfated GAG in the osteochondral regenerates ($8.8 \pm 3.6\%$ vs. $6.0 \pm 5.6\%$; $p < 0.03$) was significantly higher in the GAG group. In both groups, the GAG amount in the cartilage of the operated knee was significantly higher than in the non-involved knee ($p < 0.05$). Histological analysis of the regenerates in the GAG group was superior in comparison with the placebo group. For the first time, a biological effect following oral supplementation with GAG was demonstrated in healing of osteochondral defects *in vivo*. These findings support the known positive clinical results.

Key words

Cartilage • Glycosaminoglycans • Oral supplementation • Osteochondral lesion

Introduction

Typically, damage to the joint cartilage in major joints usually leads to osteoarthritis (OA) and thus causes

higher health costs as well as employment and salary limitations for a given socio-economic group of the society (MacLean *et al.* 1998, Steinmeyer 2000). While older patients with osteoarthritis profit from joint

replacements, treatment of younger patients remains uncertain due to the limited life of the prosthesis (Sharkley *et al.* 2002). Despite the worldwide scientific effort the possibilities of treatment of cartilage damage that have been presented so far have still not been optimized (Martinek and Imhoff 2003).

Cartilage lesions are most often treated in a conservative manner (Erggelet *et al.* 2000, Steinmeyer 2000, Towheed 2002). Very often, only the antiinflammatory effect of drugs is employed, but the basic causes of joint pathology are not decisively solved by this approach (Steinmeyer 2001). During the last 10 to 15 years, the basics of peroral medical therapy have been enriched by new aspects of OA research (Erggelet *et al.* 2000, Martinek and Imhoff 2003). Aside from NSAID, various other substances have been found to display a potential chondroprotective effect in the treatment of osteoarthritis (Debi *et al.* 2000, McAlindon *et al.* 2000ab, Reginster *et al.* 2001, Brief *et al.* 2001, Raynauld *et al.* 2002, Towheed 2002).

Various agents, including GAG (chondroitin sulfate, D-glucosamine sulfate, hyaluronic acid), piroxicam, ademetionin, oxaceprol and heparinoids (Steinmeyer 2000) have been shown to exert not only an anabolic effect on chondrocytes but also an inhibitory function on free catabolic metabolites under *in vitro* and *in vivo* conditions (Harmand *et al.* 1987, Bassleer *et al.* 1992, Vivien *et al.* 1993, Homandberg *et al.* 1997, Ronca *et al.* 1998, Uebelhart *et al.* 1998, Towheed 2002). However, until now, it has not been possible to directly prove a causal link between orally applied agents and the structural changes within the hyaline cartilage or within cartilage regenerates (Steinmeyer 2000, Brief *et al.* 2001). In spite of being supported by positive clinical results, these substances are discussed controversially and are not accepted as OA-modifying drugs (Altman and Moskowitz 2002, Felson and Anderson 2002).

GAG, whose positive effect on chondrocyte metabolism is well known, have meanwhile become prevalent in the peroral treatment of cartilage pathologies (Vidal *et al.* 1978). In recent controlled, randomized double-blind prospective studies, significant reduction of symptoms and positive radiological changes in knee joints have been demonstrated in patients with osteoarthritis following long-term GAG supplementation (Reginster *et al.* 2001, Pavelka *et al.* 2002). However, there has been no breakthrough in osteoarthritis therapy as yet. The question remains whether the peroral intake of substances such as GAG can be effective in location of

the pathology or whether they are destroyed during the digestion process.

The present animal study investigates the effect of perorally applied chondrogenic agents in the regeneration of osteochondral defects. For the evaluation, a designed preparation containing the mixture of GAG – in their natural composition – as well as vitamin E and selenium acid radical scavengers, were used to prove the biological effect in the damaged joints.

Methods

Surgery

For the experiments, 20 adult female gray Chinchilla rabbits with an average weight of 2.5 ± 0.3 kg were used and randomized into two groups. The knee joints of rabbit and man are anatomically comparable; in addition rabbits have already been used as an experimental animal for cartilage research in the knee joint (Martinek *et al.* 2002).

The procedure was performed unilaterally. Under mask anesthesia, anteromedial knee arthrotomy was performed and the patella was moved laterally. In 90° knee flexion, a 3.5 mm × 3.5 mm (width × depth) osteochondral defect was created in the trochlea femoris (Fig. 1). After thorough lavage and cleansing from residual blood the knee joint was closed and sutured in two layers.

Postoperative treatment and drug distribution

Postoperatively the animals were not immobilized and they were allowed to move freely in their cages. Prophylactic peroral antibiotic therapy with sulfonamide was conducted during the first three postoperative days. The animals were weighed once a week. Both groups received the same food but those within the GAG study group were additionally fed 50 mg/kg of the GAG-mixture with 10 µg selenium and 5 mg (10 mg/kg) vitamin E (KnorpelPlus®; Nutritect, Germany) in tablet form. The animals in the control group were given placebo tablets (milk sugar 50 mg/kg). Six weeks after the operation all animals were sacrificed. The amount and viscosity of the synovial fluid were measured and the cartilage of both knee joints macroscopically analyzed.

Sample collection and analysis

The exposed joints were photographed and biopsies (≈ 10 mg) were sampled from the tissue filled

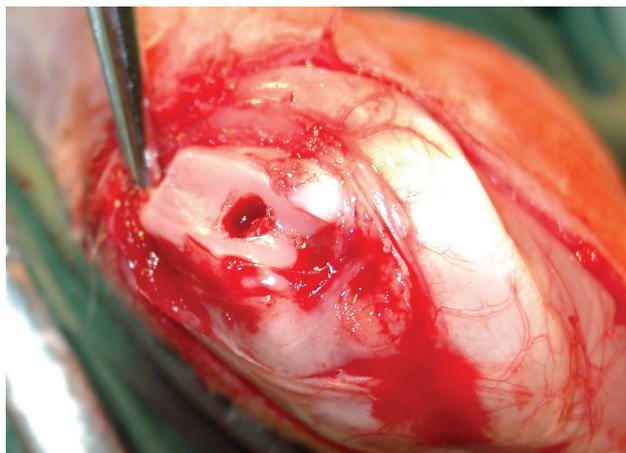


Fig. 1. Operation site: creation of the osteochondral defect in the trochlea femoris in the rabbit knee joint.



Fig. 2. The osteochondral defect after 6 weeks filled with fibrocartilage tissue placebo (**upper**), GAG group (**lower**).

inside the osteochondral defects (Fig. 2), from the surrounding cartilage and from the cartilage of the contralateral knee joint. The biopsies were analyzed for their total sulfated content as well as for the amount of N/O-sulfated GAG. The total content of sulfated GAG was determined fluorometrically (see below). The tissue was washed, lyophilized in a vacuum desiccator and the dry weight was determined. The samples were then enzymatically split into 1 ml of a 0.5 % papain-buffer solution in a 65 °C bath and 1,9-dimethylmethylene blue (DMB) was added to the aliquot. Finally, absorption was measured spectrophotometrically at 535 nm (Farndale *et al.* 1986). The GAG amount was determined by extrapolation from the standard curve with shark chondroitin sulfate (Sigma, Germany). The defects with the surrounding cartilage were removed, denatured in formaldehyde, decalcified in EDTA, cut into 5 µm layers, stained by hematoxylin/eosin (H/E) and histologically analyzed with regard to the type, orientation and number of cells as well as the character of the matrix.

Fluorescence measurements

Due to the very small volume, the viscosity of the synovial fluid was measured using a fluorescence polarization of FITC-marked polylysine as the fluorescence probe using Perrin equation for viscosity determination as described by Amler *et al.* (1990).

Non-enzymatic glycation has been estimated from the fluorescence intensity of natural fluorescent products. Steady-state excitation (emission 440 nm) and emission (excitation 335 nm or 370 nm) spectra were recorded on Perkin-Elmer LS50B spectrofluorometer. For determination of the AGEs formation dependence on the age, a 600-µl sample of the incubation solution was

diluted in 2 ml of 20 mM Tris-HCl (pH 7.4) and the fluorescence intensities of the solution at excitation/emission wavelengths 335/400 nm and 370/440 nm were recorded at 37 °C. Both excitation and emission band passes were set to 5 nm, the signal was integrated for 5 s.

The fluorescence of pentosidine and nonspecific glycation was related to the fluorescence of proteins.

Statistics

The values are shown as the mean ± S.E.M. The data were analyzed statistically by paired Student's t-test. Statistical significance was set at $p < 0.05$.

Results and Discussion

The postoperative course developed without complications in all animals. With regard to the behavior

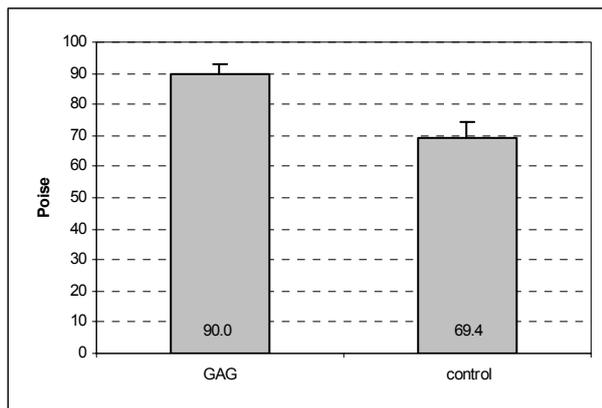


Fig. 3. Viscosity of the synovial fluid ($p < 0.001$).

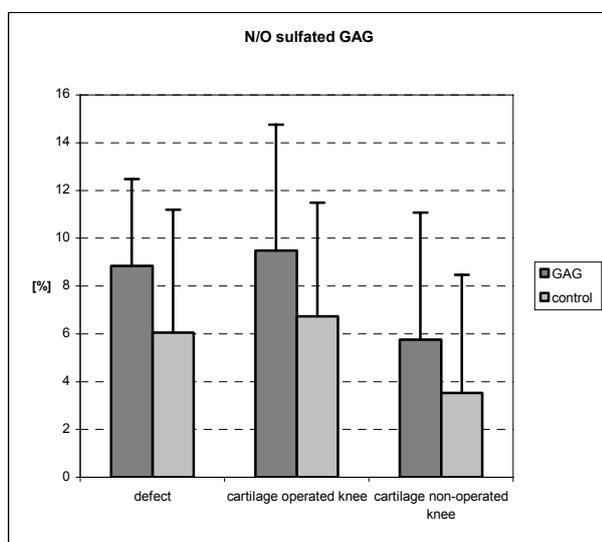


Fig. 4. The amount of sulfated GAG as share of the total weight of the regenerated tissue in the osteochondral lesions.

and mobility of the rabbits, it was not possible to discern any difference between the two groups. All animals were able to run and jump on all extremities within the whole period after surgery. In the GAG group a significantly higher ($p < 0.0004$) weight increase (1.78 ± 0.19 kg) was detected in comparison with the control group (1.13 ± 0.37 kg). The amount of synovial fluid in the control group (0.18 ± 0.22 ml) was significantly higher ($p < 0.02$) than in the GAG group (0.01 ± 0.01 ml), while the viscosity of the synovial fluid was significantly higher in the GAG group (9.0 ± 0.29 pascal-second vs. 6.94 ± 0.49 pascal-second; $p < 0.001$) (Fig. 3). Comparing the GAG group with the placebo group, the total amount of GAG content was 12.8 ± 3.6 % vs. 9.6 ± 7.8 %; ($p < 0.16$) in the osteochondral defects, 13.8 ± 6.0 % vs. 9.9 ± 5.1 %; ($p < 0.07$) in the surrounding cartilage and 8.9 ± 6.3 % vs.

5.9 ± 5.1 %; ($p < 0.15$) in the cartilage of the contralateral knee joint. The amount of the sulfated GAG in the osteochondral defects of the GAG group (8.8 ± 3.6 %) was significantly higher ($p < 0.03$) than in the control group (6.0 ± 5.6 %) (Fig. 4). In both groups the total GAG content in the cartilage of the operated knee joint was also significantly higher ($p < 0.05$) than in the non-operated knee joint.

Histological analysis showed a higher organization level of the cartilage-like repair tissue in the healed osteochondral defect in the GAG group in comparison with the placebo group.

Proteoglycans are complex macromolecules composed of numerous GAG and oligosaccharide chains which are located in the external space of the cartilage collagen network (Buckwalter and Mankin 1998). They bind water and positive ions in the cartilage substance and, in addition, form a viscous elastic layer, which lubricates and protects the cartilage surface (Brief *et al.* 2001). The presence of these aggregates is the most important factor for the biomechanical properties of the hyaline cartilage in the joints. The GAG, which are mainly composed of keratan sulfate, dermatan sulfate, heparan sulfate, chondroitin sulfate and hyaluronic acid, form the most important part of the proteoglycans (Buckwalter and Mankin 1998).

The loss of proteoglycans in the process of cartilage degeneration and osteoarthritis due to the imbalance in the exchange of matrix proteins, has a significant negative consequence on the biomechanical properties of the cartilage (O'Driscoll 1998). Theoretically, the exogenous intake of GAG can enhance the synthesis of proteoglycans in the cartilage and compensate for the loss of the matrix. This potential therapeutic effect has already been shown in clinical studies which report the improvement of clinical symptoms and radiological parameters in patients with cartilage damage or osteoarthritis by peroral doses of GAG (Reginster *et al.* 2001).

Experiments *in vitro* confirm the effect of GAG on chondrocytes (Vidal *et al.* 1978, Bassleer *et al.* 1992, Piperno *et al.* 2000). In animal studies, substances like chondroitin sulfate or glycosamine sulfate not only cause an increase in the growth rate and production of proteoglycans, but also specifically interrupt catabolic processes such as the collagenolytic activity of chondrocytes or support an antiinflammatory function in the joints (Pipitone 1991, Setnikar *et al.* 1991, Piperno *et al.* 2000).

Until now, a direct proof demonstrating structural changes in the cartilage following peroral uptake of GAG has been lacking *in vivo* (Steinmeyer 2000). Despite numerous experiments, the biological availability of peroral GAG remains uncertain (Brief *et al.* 2001). Increased levels of the hyaluronic acid and sulfated GAG in the plasma and synovial fluid can be obtained and proved in humans after intake of exogenous doses of GAG. However, the question of the required GAG concentration and the relevance of these molecules for chondropathy still remains open (Steinmeyer 2000).

In the present study it was possible to demonstrate that a continuous peroral supply of GAG mixture leads to structural changes in the regenerated cartilage in the knee joints in rabbits. Six weeks after induction of an osteochondral defect in the knee joint, an increased enrichment of sulfated GAG in the newly formed fibrocartilage was found as a result of peroral substitution with GAG. Furthermore, an increased level of total GAG was measured in the surrounding cartilage of the treated knee joint. These findings suggest that the cartilage tissue is supplied not only by blood vessels – which are responsible for the increase of GAG in the subchondral regenerate of the fibrocartilage – but also by the synovial fluid. In addition, the significantly increased viscosity of the synovial fluid could be demonstrated as a consequence of the oral GAG supplementation.

The total GAG amount in the surrounding cartilage was significantly increased in both groups and comparable between the GAG and the control group. This finding supports the hypothesis that the performed surgical intervention led to the activation of chondrocytes and consequently to an anabolic activity in the joint. According to Lotz (2001), cytokines released by the acute surgical injury were responsible for triggering this effect.

Besides these changes in the cartilage composition, the significant reduction of joint effusion in the treated joint was measured in the GAG group. However, this finding could be a function of all the KnorpelPlus-supplied agents and cannot be assigned specifically to GAG, vitamin E or selenium by this study. According to previous experiments, GAG possess an antiinflammatory effect and are able to reduce synovial inflammation (Setnikar *et al.* 1991, Ronca *et al.* 1998). In clinical studies, formation of effusions in patients is significantly diminished following peroral supplementation with GAG or chondroitin sulfate (Noack *et al.* 1994, Debi *et al.* 2000, McAlindon *et al.* 2000a).

Vitamin E is commonly known as an oxygen

radical scavenger and it has therefore been clinically used for years in various pathologies as an antioxidant agent (Grimble 1994). Selenium, an important trace element in food, is also considered to have antioxidative functions (Combs 2001). Peng and Yang (1991) described the role of selenium for the Kaschin-Beck disease, which is an endemic disease in China, with symptoms of both osteoarthritis and generalized cartilage destruction. This syndrome can be caused either by a deficit of selenium or by high levels of free radicals in the ground water (Peng *et al.* 1992). Peng and Yang (1991) were able to show that selenium significantly lowers the production of lipid peroxidase and thus serves as a protective factor for this disease. According to their study, reduction of the effusion in the treated joints in our study can be taken as a function of these oxygen radical scavengers, neutralizing aggressive cytokines.

Interestingly, the body weight of animals in the GAG-substituted group was significantly higher than that of the animals in the placebo group. This effect could be explained by an increased water content in the soft tissues and the cartilage following increased concentration of GAG. However, this hypothesis is not supported by data available in the literature since such a phenomenon has not been described before. A possible, hypothetical, explanation is the well-being with pain reduction in animals of the GAG group that leads to a higher weight increase.

The used preparation of GAG represents an important aspect in our study. The KnorpelPlus preparation contained a whole spectrum of substances that are incorporated in normal hyaline cartilage tissue and thus differed significantly from the so-called mono-preparations that are composed only of a single GAG. In our opinion, the used preparation is compatible with the hypothesis that as many building stones as possible are necessary for reconstruction of the connective tissue such as hyaline cartilage. Glycosaminoglycans contained in the KnorpelPlus were enzymatically extracted from fish cartilage to a size in the range between 1500 to 2500 Da. This enzyme portioning of glycosaminoglycans is very important for later uptake of these agents into the bloodstream and therefore for their function in the joint. Only molecules of the right size are able to pass the mucous membrane of the intestine at a high concentration and are not digested or denatured.

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