Protective effect of a new biomaterial against the development of experimental osteoarthritis lesions in rabbit: a pilot study evaluating the intra-articular injection of alginate-chitosan beads dispersed in an hydrogel

F. Oprenyeszk†, M. Chausson‡, V. Maquet‡, J.-E. Dubuc§, Y. Henrotin†||

† Bone and Cartilage Research Unit, University of Liège, CHU Sart-Tilman, Liège, Belgium
‡ KitoZyme SA, Parc industriel des Haut-Sarts, Herstal, Belgium
§ Orthopaedic Department, Cliniques Universitaires St Luc, Brussels, Belgium
|| Physical Therapy and Rehabilitation Department, Princess Paola Hospital, Vivalia, Marche-en-Famenne, Belgium

A R T I C L E   I N F O

Article history:
Received 7 January 2013
Accepted 23 April 2013

Keywords:
Osteoarthritis
Alginate
Chitosan
Hydrogel
Rabbit

S U M M A R Y

Objective: This study aimed to evaluate the structural benefit of a new biomaterial composed of alginate-chitosan (AC) beads dispersed in a hydrogel (H) derived from chitosan on the development of osteoarthritis (OA) in rabbit.

Design: OA was induced by the surgical transection of the anterior cruciate ligament in rabbits. Animals received a single intra-articular injection (900 μl) of AC beads in H hydrogel, H hydrogel alone or saline a week after surgery. OA development was followed by X-rays. Blood samples were collected throughout the study to measure biological markers (Prostaglandins E2 – PGE2 and C reactive protein – CRP).

Macroscopic observation and histological evaluation of articular cartilage and synovial membrane were performed 6 weeks after surgery.

Results: AC beads in H hydrogel prevented from the development of OA based on the reduction of the Kellgren & Lawrence (K&L) score. It also significantly reduced the histological score of cartilage lesion severity. This effect was homogenous on every joint compartment. It was due to a significant effect on cartilage structure and cellularity scores. The injection of AC beads in H hydrogel also tended to reduce the synovial membrane inflammation. No significant variation of biological markers was noted.

Conclusions: The present pilot study provides interesting and promising results for the use of AC beads in H hydrogel in animal. It indeed prevented the development of OA cartilage lesions without inflammatory signs. The potencies of this biomaterial to protect OA joint should be further documented. It could then represent a new alternative for viscosupplementation in human OA management.

© 2013 Osteoarthritis Research Society International. Published by Elsevier Ltd. All rights reserved.

Introduction

Osteoarthritis (OA) is a complex, multifactorial disease that affects a whole organ, the joint1,2. This disease remains with no cure. The principle of its treatment relies on the control of symptoms, i.e., inflammation and pain. Cartilage degeneration is one of the main features that cause the loss of articular function in OA3. It is also accompanied by the inflammation of synovial membrane and the sclerosis of subchondral bone. Based on the available therapeutics, the main recommendations for the management of OA consist in the combination of non-pharmacological and pharmacological modalities in order to control OA symptoms4–8. In fact, the ideal treatment for OA would not only control the symptoms but also prevent, slow down or stop the degradation of the joint.

Viscosupplementation (VS) is a recommended intervention9,10 that proposes through an intra-articular injection to deal with OA symptoms and to preserve the joint function. It is characterized by a delayed onset of action and the prolonged duration of the symptom relief. Indeed, the synovial fluid protects the articular surface by limiting the axial forces and reducing the friction between the cartilage surfaces. It has elastic and viscous properties due to its hyaluronic acid (HA) content. However, even if the synovial fluid is more abundant in OA, HA has an altered structure which leads to a
decrease of native properties of synovial fluid. Therefore, the injection of HA was for the past years, the VS of choice. However, one may consider VS with other compounds or biomaterials. Depending on the used product, one may foresee the prevention against cartilage degradation and/or the restoration of the articular function.

VS has to be performed with a compound that has to be biocompatible with the cartilaginous tissue, weakly biodegradable, nontoxic and not immunogenic. Natural compounds such as alginate, chitosan or collagen were shown to be suitable materials for cartilage regeneration. Indeed their structure related to that of glycosaminoglycans (GAGs) mimics the natural environment in the cartilage extracellular matrix and stimulates chondrogenesis in vitro.

Chitosan is a linear natural polymer of β-glucosamine with a variable frequency of N-acetyl-β-glucosamine units obtained by N-deacetylation of chitin which is the most abundant natural polymer after cellulose. It is present in the cell walls of fungi and the exoskeleton of shellfishes and insects. Chitosan is an interesting candidate for biomaterial that could be used for VS. Indeed, it has relevant biological proprieties such as biocompatibility, progressive degradability, absence of toxicity, lack of allergenicity and antibacterial activity. A chitosan–hyaluronate hybrid gel was recently proven to delay the progression of OA in rat meniscectomy model. Moreover, in vitro studies have shown that chitosan promoted the expression of cartilage matrix components by chondrocytes and reduced the production of inflammatory and catabolic mediators. These studies suggested that chitosan could be a good biomaterial for cartilage tissue engineering. In addition, the mix with alginate could provide the spherical shape to the biomaterial and allow the homogenous distribution of chitosan in the matrix.

The aim of the present work was to evaluate the effect of a unique intra-articular injection of a new biphasic biomaterial consisting in a mix of alginate-chitosan (AC) beads in a chitosan-derived thermogelling (H) hydrogel on the cartilage lesion in a model of OA in rabbit. One wonders if the injection could prevent from the development of OA cartilage lesion. The effects of this new biomaterial were compared to the effects of H hydrogel alone and saline.

Method

Test products

To prepare AC beads, 1.33% (w/v) chitosan and 1.85% (w/v) alginate solution were prepared separately by dissolving 1 g of ultrapure medical-grade chitosan Kiomedine-CSU (kindly provided by KitoZyme S.A.; Herstal, Belgium) in 75 mL 0.166 M acetic acid and 1 g alginate (Pronova UP LVM; FMC BioPolymer AS, Norway) in 54.1 mL 0.123 M NaOH. The two solutions were then mixed to obtain 0.5% (w/v) chitosan and 1.4% (w/v) alginate solution. Chitosan was extracted from A. bisporus edible mushrooms and characterized by a molecular weight of 42,000 Da and 20.7 mol % of degree of acetylation. The pH, measured with a stroboscopic lamp was set at the same frequency used for the nozzle, in order to visualize the falling droplets. The droplets fell into a 102 mm CaCl2 solution (Sigma–Aldrich, Bornem, Belgium). After instantaneous gelation the beads were incubated for 10 min in the CaCl2 solution and washed with saline solution. The AC beads with a diameter between 600 and 900 μm were then mixed before intra-articular injection in a thermogelling chitosan-derived (H) hydrogel which was generously provided by KitoZyme SA. H hydrogel is composed by ultrapure medical-grade chitosan Kiomedine-CSU which is characterized by a molecular weight of 136,000 Da. At room temperature, H hydrogel viscosity was 25 cP however at 37°C, viscosity increased to 1,000 cP (measured with a Brookfield LVDV-II + PRO viscometer using a spindle 18 (Brookfield Engineering Laboratories, Middleboro, Massachusetts, USA)). The pH of H hydrogel was 7.48 ± 0.03. The new biphasic biomaterial consisted in a homogeneously mix with a ratio of 1/1 of AC beads and H hydrogel.

Animals and treatment groups

OA was surgically induced by the transection of the anterior cruciate ligament (ACLT) as previously described in 21 Hyla albinus rabbits (21 weeks, weighing 3.4 ± 0.3 kg) at the time of surgery. Animals were randomly divided into three groups after surgery: Group I (n = 7): saline (control); Group II (n = 7): H hydrogel alone (H); and Group III (n = 7): AC beads in H hydrogel (AC + H). The test compounds (0.9 ml) were administered intra-articularly in the right operated knee one week after surgery. Animals were euthanized 6 weeks after surgery. All these interventions were performed by AGINKO Research AG (Marly, Switzerland). This study was approved by the Institutional Animal Care and Use Committee (IACUC) and the Committee for Animal Protection of the Ministry of Industry and Trade.

Blood sampling and serum levels of biological markers

Blood samples were collected at AGINKO facilities from each animal the day of surgery, the day of the injection and prior to sacrifice. Serum was obtained from these samples and stored at −20°C until analysis. Prostaglandins E2 (PGE2) were measured by an ELISA detection kit according to the manufacturer’s recommendations (DetectX Prostaglandin E2 High Sensitivity immunoassay; Arbor, Michigan, USA). Results were expressed in pg/ml. C reactive protein (CRP) was measured by immunoturbidimetry according to the manufacturer’s recommendations (DiAgam, Ghislenghien, Belgium) using ARCHITECT (Abbott, Rungis, France). Results were given in mg/ml.

X-rays

X-rays from the right knee were performed by AGINKO just before surgery, at the time of injection and at sacrifice. Pictures were acquired in extension. They were read by a radiologist blinded to the treatment groups and scored according to the Kellgren and Lawrence (K&L) scale. Briefly, the score evaluate the presence of osteophytes and the joint narrowing using a scale from 0 to 4, where Grade 0 corresponded to no osteophytes; Grade 1, doubtful osteophytes; Grade 2, minimal osteophytes, possibly with narrowing, cysts and sclerosis; Grade 3, moderate or definite osteophytes with moderate joint space narrowing; and Grade 4, severe with large osteophytes and definite joint space narrowing.

Macroscopic evaluation

Right after sacrifice, the right knee was dissected and further analyzed for its macroscopic appearance. To this aim, femoral condyles and tibial plateaus were colored in India ink according to the OARSI recommendations. India ink uptake was scored using a scale from 1 to 4, where Grade 1 corresponded to no uptake of India ink, which indicated an intact surface; Grade 2, minimal focal
uptake of India Ink, which indicates a minimal fibrillation; Grade 3, evident large focal dark patches of ink uptake, which indicates overt fibrillation; and Grade 4, large general uptake of India Ink, which indicates erosion of cartilage.

**Histological evaluation of cartilage**

Cartilage samples were processed for the histological evaluation after the macroscopic evaluation. Femoral condyles and tibial plateaus were fixed in 10% neutral buffered formalin and further embedded in paraffin after being decalciﬁed in DC2 medium (Labonord, Templemars, France). Sections (5 μm) from the weight bearing zones of the femoral condyles and tibial plateaus were stained in Safranin-O/Fast green and toluidine blue. The lateral and medial compartments were evaluated separately. The histological evaluation of cartilage was done according to the OARSI recommendations\(^\text{23}\). The severity of cartilage lesion was evaluated using the scale provided by the latter mentioned guidelines. Briefly, the evaluation considered the staining of the cartilage matrix (0–6), the structure of cartilage (0–11), the chondrocyte density (0–4) and the cluster formation (0–3), where 0 represented a normal situation. The maximum severity score was 24.

**Histological evaluation of synovial membrane**

Synovial membrane sampling was performed in the peri-patellar region. Tissue samples were ﬁxed in 10% neutral buffered formalin. Sections (5 μm) were stained in hematoxylin/eosin according to a standard protocol. The histological evaluation of synovial membrane was done according to the OARSI recommendations\(^\text{23}\). The inﬂammation of synovial membrane was evaluated using the scale provided by the latter mentioned guidelines. Briefly, the evaluation considered the synoviocyte proliferation (0–3) and hypertrrophy (0–3), the inﬂammatory inﬁltrate considering the lymphoplasmacytic inﬁltrate (0–3) and aggregate/follicle (0–2), the synovial stroma considering the villous hyperplasia (0–3), the proliferation of ﬁbroblast/ﬁbrocytes (0–3), the proliferation of blood vessels (0–2), the presence of cartilage or bone detritus (0–2) and hemosiderosis (0–2), where 0 represented a normal situation. The maximum severity score was 24.

**Statistical analysis**

Data are given as mean (95% conﬁdence interval) in the text and presented in box and whisker plot in the ﬁgures. Data were considered independently for the purpose of statistical analysis. They were analyzed and compared using a one-way ANOVA followed, if positive, by the Bonferroni multiple comparison post-test. \(P\)-values were considered signiﬁcant when \(P < 0.05\).

**Results**

**Safety**

Twenty-one rabbits were included in this study. One rabbit died at the time of surgery when anesthesia was initiated. Thus, group II was thereafter composed of six animals, whereas groups I and III contained seven rabbits. All animals were subjected to a daily physical examination during the study. Signs of toxicity, mortality, abnormality, and food intake were monitored. No apparent sign of toxicity were recorded.

**Biological markers of inﬂammation**

PGE\(_2\) and CRP were chosen to follow the inﬂammatory state of animals throughout the study. The variation of their serum levels are presented in Table I. PGE\(_2\) levels were increased one week after surgery in the three groups. However, PGE\(_2\) tended to decrease after injection of the test products or saline, when measured at the time of sacrifice. CRP slightly increased after surgery in group I and II while group III experienced a higher increase (Table I). At the time of sacrifice, CRP level was decreased and returned to the level measured before surgery in group I (Control, saline), increased in group II and remained stable in group III in comparison with the levels measured before injection of the test products. None of these variations were statistically significant.

**X-rays**

Standard radiographs were taken before and 6 weeks after the ACLT surgery. Representative images from each treatment group are illustrated on [Fig. 1(A)] and the individual score for each animal are presented Table II. X-rays from each animal were graded independently [Fig. 1(B)]. The X-ray score in the animals from Group III (AC + H) was signiﬁcantly less important than the one of the animals treated with saline (control) (1.5 (1.1–2.0) vs 3.0 (1.1–4.0) respectively, \(P = 0.012\), whereas the score of the animals treated with hydrogel alone (Group II, H) exhibited an intermediate value (2.2 (0.9–3.4)). However, the difference between groups I and II was not signiﬁcant.

**Macroscopic observation**

The macroscopic observation of the femoral condyles and tibial plateaus revealed the presence of cartilage lesion in the weight

---

**Table I**

<table>
<thead>
<tr>
<th>Serum levels of biological markers, PGE(_2) and CRP throughout the study</th>
<th>Before ACLT surgery</th>
<th>Before I.A. injection (1 week after surgery)</th>
<th>At the time of sacrifice (6 weeks after surgery)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>PGE(_2) (pg/ml)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group I (Saline (control))</td>
<td>514 (288–741)</td>
<td>1,268 (1,020–1,516)</td>
<td>1,032 (520–1,544)</td>
</tr>
<tr>
<td>Group II (H)</td>
<td>1,197 (491–1,902)</td>
<td>3,476 (337–6,615)</td>
<td>2,211 (383–4,040)</td>
</tr>
<tr>
<td>Group III (AC + H)</td>
<td>651 (65 to 1,367)</td>
<td>1,528 (139–2,918)</td>
<td>1,293 (283–3,204)</td>
</tr>
<tr>
<td><strong>CRP (mg/ml)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group I (Saline (control))</td>
<td>5.7 (3.1–14.47)</td>
<td>6.7 (2.2–11.1)</td>
<td>5.1 (0.6 to 10.9)</td>
</tr>
<tr>
<td>Group II (H)</td>
<td>5.8 (0.5 to 12.2)</td>
<td>6.8 (1.4–12.2)</td>
<td>12.7 (8.0 to 33.4)</td>
</tr>
<tr>
<td>Group III (AC + H)</td>
<td>2.2 (1.0–3.5)</td>
<td>14.4 (7.5–21.3)</td>
<td>15.4 (1.8–32.6)</td>
</tr>
</tbody>
</table>

Data are mean (95% conﬁdence interval). PGE\(_2\) and CRP were assayed as described in the Method section. Data were analyzed with an ANOVA followed by the Bonferroni multiple comparison test. The analysis revealed no difference between groups. I.A.: intra-articular injection of the test products or saline; H: H hydrogel alone; AC + H: AC beads in H hydrogel.
bearing zones [Fig. 2(A)]. The damaged cartilage specifically fixed the India ink. The global observation of the samples from each treatment group tended in favor of fewer lesions in the group III (AC + H) (2.0 (1.6\textsuperscript{e}2.4)) in comparison to the other two groups (Group I, saline: 2.3 (1.9\textsuperscript{e}2.8); Group II, H: 2.5 (2.0\textsuperscript{e}3.0)). This observation has been further investigated with the histological analysis.

**Histological evaluation of cartilage lesions**

The cartilage from femoral condyles and tibial plateaus was evaluated. Representative sections are illustrated [Fig. 2(B)]. The global score [Fig. 3(A)] that refers to all the compartments of the knee joint (medial and lateral femoral condyles and tibial plateaus) showed that Group III (AC + H) had a cartilage of significant better quality. Indeed, the score for this group was significantly less important (11.0 (9.5\textsuperscript{e}12.5); \(P < 0.001\) vs I (saline) and \(P = 0.001\) vs II (H)) than the score of the two other groups (I (saline): 14.8 (13.5\textsuperscript{e}16.1); II (H): 14.4 (13.2\textsuperscript{e}15.6)). The observation of the detailed score for each evaluated parameter [Fig. 3(B)] highlighted the fact that the significant difference between the treatment groups came from...
a better cartilage structure and less important changes in cellularity. The effect on cellularity was not only due to the chondrocyte density but also to the less abundance of clusters.

Finally, the analysis of the histological score considering the different compartments separately (medial and lateral or femoral condyles and tibial plateaus) revealed that the observed effect with \( \text{AC} + \text{H} \) in Group III was homogenous (Table III). No significant difference was observed between the different compartments with the three treatments.

**Histological evaluation of synovial inflammation**

The synovial membrane was evaluated in the peri-patellar region. The histological score obtained for the three groups is illustrated Fig. 4. Mild synovitis was observed in the three groups. The evaluation of Group I (Control, saline) revealed a total score of 11.0 (8.2–13.8) (maximal possible score 24). It was characterized by the synovial lining hypertrophy and the presence of inflammatory infiltrate. As shown on Fig. 4, both treatment groups tended to decrease the synovial membrane histological score (Group II: 10.5 (7.0–14.0) and Group III: 9.5 (7.0–12.2)) even if the statistical significance was not reached.

**Discussion**

This study demonstrated the potential of a new biomaterial that consisting in a mix of AC beads in chitosan-derived thermogelling (H) hydrogel to protect against the development of cartilage lesion.
in a rabbit model of OA. The present results indicated a strict difference between the injection of H hydrogel alone or with AC beads. Indeed, X-rays indicated that the injection of AC beads in H hydrogel prevented joint space narrowing and based on the histological evaluation, the intra-articular injection of this new biomaterial significantly reduced the severity of cartilage lesion. In addition, the intra-articular injection of AC beads in H hydrogel did not produce any additional sign of inflammation. This difference could be explained by the mechanical properties of the beads that would be able to increase the viscosity of the hydrogel then decreasing the friction coefficient between joint surfaces and absorbing strains applied on the joint during movement.

The ACLT rabbit model of OA as it was previously described was chosen for the present pilot study as it is a suitable model for the evaluation of the structural effects of a therapy. The macroscopic observation of the different compartment of each animal confirmed that the ACLT surgery produced cartilage lesions that were further quantified by the histological study. The cartilage lesions observed in this study in the saline (control) group, 6 weeks after the ACLT surgery, were moderate to severe. OA damages were visible on the structure of cartilage (i.e., fissure, pitting and cartilage loss) as well as on the quality of the extra-cellular matrix and on cellularity. The injection of AC beads in H hydrogel was able to affect cartilage structure as well as cellularity, whereas H hydrogel alone produced no effect. Of note, osteophytes were also observed on the femoral condyles, tibial plateaus and trochlea but no difference was observed between the three groups (data not shown).

**Fig. 3.** Cartilage histological score and comparison between treatment groups (Group I: saline (control); Group II: H hydrogel alone (H); and Group III: AC beads in H hydrogel (AC+H)). (A) Cartilage global score; (B) Detailed global score: values for the evaluated parameters. Data are presented as a box and whisker plot. Data were analyzed with an ANOVA followed by the Bonferroni multiple comparison test. *P vs saline (control); #P vs H hydrogel alone (H). N = 7 for groups I and III, N = 6 for group II.
These first results were encouraging for the use of this new biomaterial for VS in OA for its potential structure-modifying effect. However, one may keep in mind that the use of young animal could yield to a better structure-modifying effect. In addition, one may emphasize that no direct extrapolation from any kind of animal model of OA can be done. So these results will have to be confirmed in human.

Surgery induced an inflammatory reaction as shown by the increase of CRP and PGE₂, two markers of inflammation. No additional signs of inflammation were noted after the intra-articular injection of the test products. Furthermore, the histologic evaluation of the synovial membrane showed that the intra-articular injection of AC beads in H hydrogel was associated with a decrease of the synovial inflammation. Synovial inflammation is known to echo the degenerative process and the disease progression. The fact that the intra-articular injection of AC beads in H hydrogel tended to reduce the histological score of the synovial membrane could mean that this treatment helped to contain the degenerative process that occurs along OA development. This argument could be sustained by the observations made with the cartilage histology on the cellularity and the structure of cartilage. Indeed, AC beads in H hydrogel improved cellularity and reduced the presence of clusters in cartilage. The development of clusters is a well-characterized event of OA pathogenesis. They are the results of an extensive proliferation of chondrocytes that lead to chondrocyte hypertrophic differentiation and cell death. Thus, the reduction of cluster in OA cartilage due to the presence of AC beads in H hydrogel could prevent from cartilage degradation and then preserve its structure. Altogether these results are in favor of a structure-modifying effect of AC beads in H hydrogel.

HA has been widely used for VS. It was described to alleviate OA symptoms and even produce structural effect. It was also observed in rabbit in a meniscectomy model of OA. Chitosan and alginate could be candidates for VS. Alginate is required to maintain the spherical form of the biomaterials. As HA, chitosan is a natural polysaccharide with a chemical structure close to that of GAGs. They are also of non-animal origin. The results of the present work showed that VS with the new biphasic biomaterial could protect the articular cartilage from degradation.

VS has been employed in OA patients for many years. VS with HA has been shown to delay the total knee replacement in OA patient. One may expect even better results with biomaterials that would be better tolerated by patients. In addition, the AC beads in H hydrogel could be used not only for VS in OA patients but also as drug delivery system or cell carrier for transplantation. The studied biomaterial presents all the characteristics to fulfill the pre-requisites for these two applications. Chitosan has already been proposed for this kind of use. These matters should be further investigated.

This study consisted in a first proof of concept toward the development of a new product for VS applied to the osteoarthritic joint. It was performed in a small number of animal and with a single formulation of the biomaterial. It aimed at first testing the potential of this new biomaterial. This research has to be further pursued. Cartilage lesion in the ACLT rabbit model are usually evaluated at 8 or 12 weeks. The joint has been evaluated in the present study, 6 weeks after surgery. Indeed, the choice was made to evaluate the prophylactic effect of the intra-articular injection performed 1 week after surgery on the development of the lesions in early OA, since it was described in the first 8 weeks of the model. Hence, the encouraging results that were obtained here are in favor of the protective effect of this biomaterial, have to be further described. The precise mechanism that leads to the structure-modifying effect observed with the intra-articular injection of the biomaterial should be investigated. One may foresee a mechanical effect through the beads themselves, since the injection of hydrogel alone does not produce any significant effect. Indeed, these results are in the line of our previous observation in a pilot experiment testing the mechanical properties of this biomaterial in osteochondral defect in rabbit where AC beads resist to mechanical stress generated by the animal locomotion (personal communication). Finally, as usually mentioned with VS, the remanence of the test product has to be investigated.

**Conclusion**

The present pilot study provides interesting results supporting the development of the new biphasic biomaterial, AC beads in chitosan-derived thermogelling hydrogel for the intra-articular treatment of OA in human. It indeed prevented the development of OA cartilage lesions without additional inflammatory signs. The potencies of this biomaterial to protect OA joint should be further documented. It could then represent a new alternate for VS in human OA management.

**Author contribution**

- Conception and design: all authors.
Acknowledgments

The authors would like to thank Christelle Boileau for her kind assistance in this manuscript preparation and Chantal Hunblet and Geraldine Piel for their technical assistance. The authors are grateful to the “Région Wallonne” for the FIRST Post-Doc program for the CARTIMAT project.


