The comparison of the effects of a novel hydrogel compound and traditional hyaluronate following micro-fracture procedure in a rat full-thickness chondral defect model

Yunus Emre Akman,*, Erhan Sukur, Ahmet Senel, Nur Ece Oztas Sukur, Canan Kelten Talu, Yusuf Ozturk

a Metin Sabanci Baltalimani Bone Diseases Training and Research Hospital, Department of Orthopaedics and Traumatology, Istanbul, Turkey
b University of Sakarya, Sakarya Training and Research Hospital, Department of Orthopaedics and Traumatology, Sakarya, Turkey
c Istanbul Education and Research Hospital, Department of Pathology, Istanbul, Turkey
d University of Sakarya, Sakarya Training and Research Hospital, Department of Pathology, Sakarya, Turkey
e Istanbul Education and Research Hospital, Department of Pathology, Istanbul, Turkey

Article history:
Received 22 October 2016
Received in revised form 9 December 2016
Accepted 10 April 2017
Available online 13 June 2017

Keywords:
Cartilage lesion
Micro-fracture
Hyaluronate
Chondroitin sulfate
N-acetyl-D-glucosamine

Purpose: The aim of this experimental study was to investigate the impact of HA–CS–NAG compound (hyaluronate, sodium chondroitin sulfate, N-acetyl-D-glucosamine) on the quality of repair tissue after micro-fracture and to compare it with HA (hyaluronate), in a rat full-thickness chondral defect model.

Methods: Full-thickness chondral defects were created in a non-weight bearing area by using a handle 2.7-mm drill bit, in the right knees of 33 Sprague–Dawley rats. Each specimen then underwent micro-fracture using a needle. Two weeks after surgery, 3 groups were randomly formed among the rats (n = 33). In Group 1, 0.2 mL of sterile saline solution (0.9%) was injected. In Group 2, 0.2 mL HA with a mean molecular weight of 1.2 Mda was injected. In Group 3, 0.2 mL of HA–CS–NAG compound (hyaluronate, sodium chondroitin sulfate, N-acetyl-D-glucosamine) was injected. The injections were applied on the 14th, the 21st and the 28th postoperative days. All rats were sacrificed on the 42nd postoperative day. Histological analysis of the repair tissue was performed for each specimen by two blinded observers using Wakitani scoring system.

Results: There was significantly improved repair tissue in both Group 3 and Group 2 when compared with Group 1. Group 3 showed statistically significant improvement in terms of ‘cell morphology’ and ‘integration of donor with host’ when compared to Group 2 (p < 0.001).

Conclusion: Intra-articular injection of HA–CS–NAG compound after micro-fracture results in significantly improved repair tissue in rats’ chondral defects when compared to HA regarding the donor integration and cell morphology.

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Introduction

Full-thickness cartilage defects are common and related to trauma or overuse. Due to low cellular activity of chondrocytes and the absence of vascular response, they have very limited capacity for self-repair. An ideal treatment for cartilage lesions aims to recreate a healthy hyaline cartilage in the area of the defect that is integrated with surrounding normal cartilage and has similar mechanical properties.1

Micro-fracture is one of the marrow stimulation techniques and often used to treat focal full-thickness cartilage defects (2.5 cm ) due to simple and minimally invasive application properties, lower morbidity and cost-effectiveness.2–4 The short-term results are satisfying with this technique. Although some long-term studies have reported good results and little decline in the functional improvement seen after micro-fracture3,4 some other studies reported long term results to be poor and resulting in osteoarthritis
regardless of the defect size. Therefore there is still a concern about the durability and longevity of the fibrocartilaginous repair tissue. The main nature of repair tissue after micro-fracture is fibrocartilage, which primarily contains type I collagen and can't fully replicate the biomechanical features of hyaline cartilage, and probably this would be the reason for long term failure of micro-fracture technique. Thus, with the aim of generating pure quality of hyaline cartilage without fibrous and hypertrophic tissues, the management of long-term functional healing still remains challenging and many pharmacologically active drug molecules are under investigation in order to increase the quality and the endurance of the fibrocartilaginous repair tissue obtained by the micro-fracture procedure.

HA supplementation has been suggested as a supportive agent after micro-fracture. However, supplementation with HA combined with structure modifying drugs including CS and glucosamines which are thought to be potentially effective as chondroprotectives by altering the catabolic and anabolic balance of the joint has not been evaluated yet. These agents are candidates for intra-articular use, due to either their poor oral bioavailability or concern for systemic toxicity. In a previous study, it is found out that a recently introduced intra-articular injectable hydrogel form of a compound of HA (hyaluronan) (36 mg/2.25 mL), CS (chondroitin sulfate) (45 mg/2.25 mL) and NAG (N-acetyl-D-glucosamine) (9 mg/2.25 mL) is more chondroprotective to rats' cartilage when compared to HA during the early stages of osteoarthritis with respect to preserving and healing articular surfaces, cellular abnormality and proteoglycan content. Despite of the presence of many studies on the intra-articular use of HA, oral NAG and CS combination, there is no experimental data to evaluate the intra-articular application following micro-fracture application and the effects on hyaline cartilage of these drugs. The purpose of this experimental study was to compare the effects of intra-articularly injected HA and intra-articular injectable hydrogel form of a compound of HA, NAG and CS which are applied following microfracture procedure in an full-thickness chondral defect model in rat, through the histopathological parameters.

The purpose of the present study was to investigate the impact of HA—CS—NAG compound viscosupplementation on the quality of repair tissue after micro-fracture and to compare with the traditional HA in a rat full-thickness chondral defect model.

Materials and methods

Study design

The animal experiment was approved by the Institutional Animal Care and Use Committee. Thirty three adult female Sprague−Dawley rats (approximately 250−350 g, 12 months old) were used. All animals were housed in standard cages, five animals per cage at 20−24 °C and at 50−60%, and a standard 12-h light and 12-h dark cycle was used after the operation. The animals were fed with rat chow and water ad libitum.

The rats were anesthetized with a combined intra-peritoneal injection of ketamine (50 mg/kg) and xylazine (5 mg/kg). The right limbs of the rats were shaved. A medial para-patellar incision was used in the right knees of the rats under sterile conditions. After the lateral dislocation of the patella, the articular cartilage of the patellar groove and the femoral condyles were exposed. Full-thickness chondral defects in the articular cartilage of the intercondylar notch were created without violation of the subchondral bone, using a handled 2.7 mm diameter drill bit. The defects were created in the articular cartilage of the intercondylar notch as this area is a non-weight bearing region of the distal femur. A non-weight bearing region was chosen because the rats were left free to move freely inside the cages, in the postoperative period. The drill tip contacted the surface vertically, cutting lips of the drill directly engaging the chondral surface. Rotational movements with slight pressure on the drill bit was applied to create a defect with a diameter of approximately 3 mm. High speed drilling was not applied to avoid thermal damage. Three holes were made using a needle in each defect as more number of holes would lead to violation of the neighboring holes. Needle tapped into the subchondral bone, avoiding thermal damage to the defects, to a depth of approximately 3 mm, until bleeding from the hole was present (Fig. 1a). The debris was completely removed. Then, the patella was reduced, and joint capsule and subcutaneous tissue were closed in anatomical layers reduced, and joint capsule and subcutaneous tissue were closed in anatomical layers with 4/0 vicryl after the operation. The same environmental and dietary conditions were provided for all animals, during the study.

Two weeks after surgery, rats (n = 33) with experimentally created full-thickness chondral defects were randomly divided into 3 groups as follows and the injections were performed in the operated knees:

Group 1: 0.2 mL of sterile saline solution (0.9%) to serve as the control group.

Group 2: 0.2 mL HA with a mean molecular weight of 1.2 Mda (Ostenil; TRB Chemedica AG, Munich, Germany).

Group 3: 0.2 mL of HA−CS−NAG compound [sodium hyaluronic acid (36 mg/2.25 mL), sodium chondroitin sulfate (45 mg/2.25 mL) and N-acetyl-D-glucosamine (9 mg/2.25 mL) (Genvisc Gplus, Phibio GmbH, Frankfurt am Main, Germany).

Injections were performed on the 14th, the 21st and the 28th days consecutively under clean conditions (Fig. 1b). All rats were sacrificed with high dose of intraperitoneal thiopental (200 mg/kg), on the 42nd postoperative day (two weeks after the last injection).

Tissue preparation and histological grading

After the sacrifice, the tibia and the femur were cut in order to separate the tibio-femoral joints of the rats. Tendons and the attaching ligaments were removed using a surgical blade. Specimens were fixed at 10% buffered formalin and decalcified in 8% formic acid. The specimens were placed in paraffin after dehydration process performed through graded series of ethanol solutions. Five micrometer sections were cut through the micro-fractured osteochondral defects sagittally, perpendicular to the defect. The specimens were stained by hematoxylin and eosin. The defects to be examined were numbered randomly and noted elsewhere. Histologic analysis of the repair tissue was performed for each specimen by two blinded observers using Wakitani scoring system which is a well-detailed histologic grading system. The scoring system is composed of five categories, including cell morphology, matrix staining, surface regularity, thickness of cartilage and integration of donor with host, and assigns a score ranging from 0 to 14 points.

Statistical analysis

The adjustment between observers was evaluated using reliability statistics two-way random absolute agreement method. Data were evaluated using SPSS for windows 21.0 software (SPSS Inc, Chicago, IL). A power analysis using pilot data was performed before beginning the study. This analysis determined that with a 95% confidence limits and a power of 80%, 11 animals per group would be required.

Descriptive statistics were calculated as frequency and percentage for categorical variables and as mean, standard deviation...
and median for numerical variables. One Way ANOVA test was applied for the comparison of independent groups. The ordinal rating system of the subgroups were compared with Kruskal Wallis test. The subgroup analyses were performed with Mann Whitney U test and Bonferroni correction was adjusted. Chi-square test was used to compare the rates in the groups. Monte Carlo simulation was performed as the required qualifications were not available. Significance level was set at 0.05.

Results

The mean total Wakitani scores were 10.82, 8.91 and 7.18 for Group 1, Group 2 and Group 3 respectively. There was statistically significant difference between Group 1 and Group 2 (p = 0.041), between Group 1 and Group 3 (p = 0.000), and between Group 2 and Group 3 (0.028) regarding total Wakitani scores.

The mean values for ‘cell morphology’ parameter were 3.18, 2.45 and 1.82 for Group 1, Group 2 and Group 3 respectively. Group 3 showed statistically significant improvement when compared with both Group 1 (p = 0.002) and Group 2 (p = 0.016). There was also significant improvement regarding ‘cell morphology’ in Group 2 when compared with Group 1 (0.042). This result statistically demonstrates that the repair tissues obtained following micro-fracture procedure in Group 2 and Group 3 were much more like hyaline cartilage compared with the repair tissue obtained in Group 1, however Group 3 was superior to all.

The mean values for ‘matrix staining’ parameter were 2.27, 1.91 and 2.00 for Group 1, Group 2 and Group 3 respectively. There was no statistically significant difference among the 3 groups regarding this parameter (p > 0.05). This result demonstrates that there was markedly reduced metachromatic staining with toluidine blue compared with neighboring uninjured cartilage.

The mean values for ‘surface regularity’ parameter were 2.00, 1.73 and 1.55 for Group 1, Group 2 and Group 3 respectively. There was no statistically significant difference among the 3 groups regarding this parameter (p > 0.05). This finding suggests that the surface of the defect was irregular or moderately regular following the treatment, compared with the entire chondral surface, in all groups.

The mean values for ‘thickness of cartilage’ parameter were 1.73, 1.45 and 1.18 for Group 1, Group 2 and Group 3 respectively. There was no statistically significant difference among the 3 groups concerning this parameter (p > 0.05). Thus, the average thickness of the cartilage in the defect did not significantly differ among the 3 groups.

The mean values for ‘integration of donor with host’ parameter were 1.64, 1.36 and 0.64 for Group 1, Group 2 and Group 3 respectively. Group 3 showed statistically significant improvement in terms of ‘cell morphology’ when compared with both Group 1 (p = 0.003) and Group 2 (0.013). However, there was no statistically significant difference between Group 1 and Group 2 (p = 0.211). This result statistically demonstrates that the obtained chondral tissue after the treatment integrated with the host adjacent cartilage best in Group 3 when compared with Group 1 and Group 2.

Figs. 2–4 demonstrate photomicrographs of the full-thickness defects after the treatment in Group 3, 1 and 2 respectively. Mean scores of the Wakitani scoring system parameters of each group after the treatment and statistical evaluation with the comparison of more than two independent groups, made by One Way Anova test (F test), are demonstrated in Table 1. Table 2 demonstrates

Fig. 2. a) The defective area in a specimen belonging Group 3, composed mostly of fibrocartilaginous tissue with superficial depression, is seen in the upper right corner of the picture. b) Toluidine blue staining shows complete loss of matrix metachromasia in the defective area.
intergroup comparisons of the Wakitani scoring system parameters.

**Discussion**

In our experimental full-thickness chondral defect model in rat, together with an incomplete defect fill, a thin repair tissue with an irregular surface and poor adjacent area integration were observed in histologic evaluation of control specimens treated with micro-fracture alone. Evaluation of the specimens in which intra-
articular HA was applied following the micro-fracture procedure demonstrated improvement of the repair tissue within the defect histologically. The scores of the group in which HA—CS—NAG compound was injected, were statistically significant when compared to both the control group and the group in which HA was injected following the micro-fracture procedure, in each of subcategories of Wakitani scoring system including cell morphology, matrix staining, surface regularity, thickness of cartilage, integration of donor with host. Thus, this study shows that intra-articular injection of the HA—CS—NAG compound after micro-fracture results in significantly improved repair tissue in rats' cartilage defects when compared to HA regarding the donor integration and cell morphology.

Significantly higher histologic scores obtained with HA—CS—NAG hydrogel compound injection, may be attributed to the stimulatory effect of NAG on hyaluronic acid synthesis and the anti-inflammatory effect of CS which also reduce the apoptosis of the chondrocytes. Ando et al reported endogenous HA to increase the stimulatory effect of NAG on hyaluronic acid synthesis and the anti-inflammatory effect of NAG hydrogel compound injection, may be attributed to NAG but it might be a mechanism for the therapeutic effect of HA.23 Together with the chondroprotective effect of HA, NAG which has stimulatory effect on hyaluronic acid synthesis in human articular chondrocytes and synovial fibroblasts23 inhibits nitric oxide, cyclooxygenase-2 (COX-2), and IL-6 production, resulting with reduced apoptosis in cultured human chondrocytes.24 Some anti-inflammatory properties have also been attributed to CS, as it can inhibit leukocyte chemotaxis and phagocytosis in order to protect the plasma membrane25 and to reduce COX-2 expression and prostaglandin E2 production by the chondrocytes.26 Furthermore, CS can reduce the apoptosis of chondrocytes via mitochondrial pathway.27 CS inhibits extracellular proteases involved in the metabolism of connective tissues and stimulates proteoglycan production by chondrocytes in vitro; they also inhibit cartilage cytokine production and induce apoptosis of articular chondrocytes.28 It is also demonstrated that CS enhances the HA production by fibroblast like synoviocytes through activation of the p38 MAP kinase and Akt signaling pathways and through differential modulation of the HA synthases isozymes.29 Hui et al reported that intra-articular injection of CS carried by hydrogel at 100 mg/ml concentration has improved the biomechanical and histological properties of the repaired cartilage of chondral defects in a rabbit model.30 Intra-articular delivery of both glucosamine and CS is a feasible alternative to overcome the pharmacokinetic obstacles associated with the oral dosing. The bioavailability of CS is as low as 10–15% when it is administered orally.31 Also, oral glucosamine may increase gastric acidity leading to ‘glucosamine allergy’ (gastric irritation, wheezing, constipation and gastric ulceration).22 Moreover, oral glucosamine has serious side effects including insulin resistance, acute hepatic failure and ocular hypertension as well.32–35 These are the mainstay of various studies concerning about more effective, practical and safe administration of these molecules with demonstrated chondroprotective effects. Currently it has been shown that intra-articular HA, NAG and CS application was safe and efficacious in OA trials, experimental animal models and in vitro studies.19,36–38 In our experimental rat model, we also found out that intra-articular application of HA, NAG and CS is effective according to our histological evaluation.

In conclusion, intra-articular injection of the HA—CS—NAG compound after micro-fracture results in significantly improved repair tissue in rats' cartilage defects when compared to HA regarding the donor integration and cell morphology. Hence, with its better structure modifying and chondroprotective effects, viscosupplementation with HA—NAG—CS hydrogel compound may be considered as an alternative to traditional HA viscosupplementation after micro-fracture treatment of the chondral defects, in order to enhance the quality of the repair tissue. Future experimental and clinical studies evaluating the effect of varying the dose or frequency of HA—CS—NAG compound viscosupplementation are going to be more meaningful and required.

Acknowledgments

We thank Nilüfer Akman Dogan, PhD for her invaluable contribution in performing the statistical analysis. We also thank Bezmialem Vakif University Animal Testing Laboratory for the excellent assistance in performing our experimental study. This study was supported by Istanbul Education and Research Hospital Scientific Research Fund.

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