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In vitro and *in vivo* evaluation of doxycycline-chondroitin sulfate/PCLmicrospheres for intraarticular treatment of osteoarthritis

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Abstract: Osteoarthritis (OA) is a degenerative joint disease, which has no complete treatment with medication yet. Intraarticular hyaluronan (HA) injection can decrease pain and modify the natural course of OA. This study was designed to provide long term delivery of an MMP (matrix-metalloproteinase) inhibitor agent-doxycycline, together with matrix regenerative agent-chondroitin sulfate for treating OA which progress with matrix degenerations. Doxycycline (D) and doxycycline-chondroitin sulfate (D-CS) loaded poly-ε-caprolactone (PCL) microspheres (MS) were prepared as intraarticular delivery systems. Bio-effectiveness of developed microspheres was first evaluated with three-dimensional *in vitro* model of OA where both MS showed significant reduction in MMP-13 levels compared to untreated OA-chondrocytes at 15 and 24 days. Significant decrease was observed in GAG release into the media for both D MS and D-CS MS treated groups at 15 and 24 days. Second, the microspheres were injected to rabbit knee in hyaluronan (HA) to evaluate the effectiveness of the treatment. Radiographic scores of D MS and D-CS MS groups improved after 8 weeks when compared to OA group. Mankin-Pitzker histological scores similarly showed improvement with D MS and D-CSMS groups when compared to OA group. *Ex vivo* hardness tests of cartilages demonstrated superior hardness values with both doses of D-CSMS compared to OA group. D MS showed promising improvement of OA in histology results. Although, both MS groups had similar effects on cells in the *in vitro* model, D-CSMS had a positive contribution on all *in vivo* treatment outcomes and showed potential as a new strategy for treatment when applied to OA knee joints. © 2014 Wiley Periodicals, Inc. J Biomed Mater Res Part B: Appl Biomater, 103B: 1238–1248, 2015.

Key Words: drug delivery/release, articular cartilage, microspheres, matrix metalloproteinase, chondroitin sulfate

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INTRODUCTION

Osteoarthritis (OA) is a chronic multi-factorial disease associated with thinning and worsening of the joint cartilage.[1] It is the most common joint disease leading to pain, deformity and deterioration of function.[2] Treatments with oral drugs have various side effects and shortcomings especially in long term use.[3] Therefore, intraarticular (IA) drug injection has been developed as a new treatment method in recent years.[1,4] Hyaluronan (HA), a major component of synovialfluid and cartilage matrix, is a large, polydisperse, linear, anionic nonsulfated glycosaminoglycan composed of repeating disaccharides of glucuronic acid and *N*-acetylglucosamine.[5] Intraarticular (IA) injection of HA is known to be safe, effective and well-tolerated.[6,7] Hyaluronan alters the rate of disease progression, relieves pain and increases joint lubrication.[8] However, OA progression rate is controversial and provides only short-term pain relief with HA treatment.[5,7,9] In literature, several intraarticular injections of hyaluronan are recommended to provide a control for OA progression; but it is not known whether there is a need for repeated injections and if so, what is the ideal time between injections.[6] Because of complications, the HA treatment level is unclear.[5,10,11] So, this approach has limitations including short term pain relief and requirement of frequent injections.[6,12]

Disclosure of Potential Conflicts of Interest: This study has a patent pending (TR2012/00160) for the manufacture and use of Doxycycline and Doxcycline-Chondroitin sulfate loaded PCL microspheres for intra-articular delivery in the treatment of osteoarthritis.

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Biodegradable polymers are the most widely preferred systems in controlled release applications. Poly-ε-caprolactone (PCL) is a hydrophobic, semi-crystalline, and slow-degrading polymer that does not produce an acidic environment during biodegradation.[13] This polymer can be used in long-term delivery systems, especially for applications lasting more than 1 year.[13,14] Doxycycline (D) is a widely available, inexpensive and stable antibiotic from tetracyclines.[15] Prior studies have shown that tetracyclines, especially doxycycline has the ability to inhibit matrix-metalloproteinases (MMPs) in both *in vitro* and *in vivo* conditions.[16] Chondroitin sulfate (CS) is a glycosaminoglycan, which is naturally found in the extracellular matrix of articular cartilage.[17] Gelatin/chondroitin 6-sulfate microspheres were used in delivery of therapeutic proteins to mouse joints.[18]

In this study, we questioned whether a local treatment approach with controlled release system of an MMP inhibitor would be effective against an established OA disease either alone or when co-loaded with chondroitin sulfate. To find the answer, developed and characterized microspheres were tested with *in vitro* studies on agarose-chondrocyte 3D constructs for their efficacy on OA chondrocytes and they were compared with current local treatment method (HA injection) on experimental model of OA on rabbits by radiographic, biomechanical, and histopathology analysis.

MATERIALS AND METHODS

Preparation and characterization of microspheres

Doxycycline loaded (D MS) and doxycycline-chondroitin sulfate co-loaded (D-CS MS) PCL (Mw: 14 kDa) microspheres were prepared using polyvinyl alcohol (PVA- 4%) as an emulsifier. Preparation of these two types of microspheres is described elsewhere.[19] Briefly, during preparation of D-CS MS, the oil phase was stirred for half an hour at 3400 rpm to establish the oil in water (o/w) emulsion and reduce the size and then the stirring rate switched to 1100 rpm as in preparation of D MS. Microspheres were sterilized by gamma irradiation prior to use. Doxycycline and chondroitin sulfate release into cell culture media and encapsulation efficiencies of each type of microspheres was measured by spectrophotometer. D MS (5 mg) or D-CSMS (5 mg) was added to the cell culture media by using cell culture inserts in order to prevent loss of microspheres during sample collection at defined time points.

Experimental OA model establishment

Experimental OA was established for *in vitro* cell culture and *in vivo* studies. Animals were housed individually and maintained in accordance with the Helsinki Animal Rights and Turkish Veterinary Medicine Deontology Regulations (6343/2). The Animal Care and Ethics Committee of the Middle East Technical University (Ankara, Turkey) approved the study. Albino adult male rabbits weighing an average of 3.0 ± 0.5 kg were anesthetized with intramuscular injection of 35 mg/kg ketamine hydrochloride (Ketasol 10%, Richter-Pharma, Austria) and 5 mg/kg xylazin hydrochloride (Alfazyne 2%, Alfasan, Woerden, The Netherlands). Collagenase type II (*Clostridium histolyticum*, Sigma) was dissolved in saline and filtered through a 0.20 μm membrane. The enzyme activity of collagenase solution was adjusted to 456 U mg^{-1} solid. The injections were performed at day 1 and 4; 0.5 mL collagenase solution was injected into each hind knee joint transcutaneously by an orthopedic surgeon experienced in intraarticular injections.[20]

Chondrocyte isolation and culture

Approval from Ethical Board of Hacettepe University School of Medicine was obtained for the use of animals in cell isolation. Chondrocytes were isolated from articular cartilage of osteoarthritic knees of rabbits following the modified procedure of Ref. 21. Briefly, after 6 weeks of collagenase injections, the harvested joints were first immersed in ethanol (96%) and then the surrounding tissues were scraped off aseptically. Treated with collagenase type II (100 U mL^{-1}) for 16–17 h at 37°C, the obtained cell suspension was filtered through a sterile 100 μm cell strainer. The filtrate was centrifuged at 2000 rpm for 5 min (EBA-20, Hettich, Germany). Chondrocytes were cultured in RPMI-1640 (10% fetal bovine serum (FBS), 10 U antibiotic/ml) (Biochrom, Germany) medium at 37°C under 5% CO_2 in an incubator (5215, Shel Lab, USA). The growth medium was changed every third day and the chondrocytes were passaged with trypsin-EDTA solution (0.05%) in a 1:3 ratio. Primary and 1st passage chondrocytes were used for cell culture studies.

Three-dimensional *in vitro* osteoarthritis model

For *in vitro* experiments, a three dimensional (3D) OA agarose model was developed using chondrocytes of OA induced rabbits.[22] Briefly, chondrocytes were embedded in agarose (2% low-melting agarose-gelling temperature $25°C \pm 5°C$) (Sigma, USA) prepared in phosphate buffer solution. Then, equal volumes of chondrocyte suspension in double strength RPMI-1640 (20% FBS) were mixed with agarose to obtain a final chondrocyte concentration of 10^6 cells mL^{-1} in each well of a 24-well plate. About 1 mL of RPMI-1640 (10% FBS) was then added into each well. Medium was refreshed every 3 days. On the third day of *in vitro* cultivation, 20 ng mL^{-1} interleukin-1β (IL-1β) (Sigma–Aldrich, USA) was added into the medium. Same amount of IL-1β was added during each medium change.

Sample collection and analysis protocol

In Figure 1, the study design of the *in vitro* study is given. Establishment of the *in vitro* OA model and effectiveness of the release systems were evaluated after incubations for 9, 15, and 24 days. The samples were divided into two groups based on the presence or absence of IL-1β. For determination of the effects of microsphere treatment, IL-1β group was subdivided into three groups as; untreated and D MS (5 mg), D-CS MS (5 mg) treatments. The chondrocytes in agarose constructs cultured only in media (RPMI-FBS) without IL-1β, D MS, and/or D-CS MS, served as controls. The media were collected and stored at −80°C for GAG, hydroxyproline (HYP) and MMP-13 analyses. Agarose constructs with chondrocytes were digested with papain for GAG, HYP and DNA quantitation analyses.[22] DNA amounts of chondrocytes in agarose were used to normalize the results of GAG and collagen amounts.

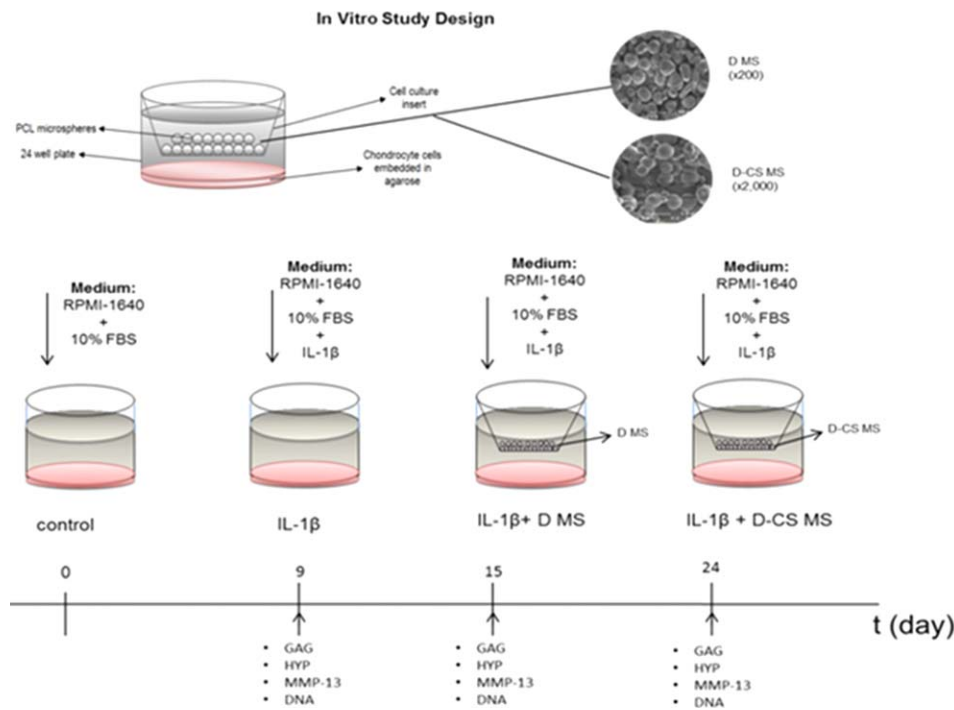


FIGURE 1. Outline of the *in vitro* study. Schematic illustration of the system used for evaluation of efficacy of microspheres in 3D *in vitro* OA model. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Glycosaminoglycan (GAG) assay

Total sulfated glycosaminoglycan (sGAG) amounts both in papain digests of agarose-chondrocyte constructs and in media were determined by using 1,9 dimethylmethylene blue (DMMB) assay.[23] Chondroitin sulfate from bovine trachea (Sigma, USA) was used as standard. Total sGAG amounts of constructs were reported as sGAG/DNA (μg/μg) and sGAG concentrations of liquid media were reported as μg/ml.[22]

Hydroxyproline (HYP) assay

The papain digests and collected media were used for hydroxyproline (HYP) assay to determine the collagen content.[24] 4-hydroxyproline (Sigma) was used as standard. HYP:collagen converting factor is reported as 1:8 in literature. HYP content of constructs was reported as HYP/DNA (μg/μg) and HYP concentrations of liquid media were reported as μg mL^{-1}.[22]

DNA assay

DNA amounts of papain digested samples were determined by Hoechst 33258 dye (Invitrogen, Germany) with Fluorometer (Modulus, USA). Calf thymus DNA was used as standard. The measurement was performed according to the protocol of the instrument and DNA concentrations were measured as (μg).

Matrix metalloproteinase-13 (MMP-13) assay

MMP-13 concentrations in cell culture media were quantified by enzyme linked immunosorbent assay (ELISA) (Cusabio) according to the protocol of MMP-13 kit. MMP-13 concentrations were measured as ng mL^{-1}.

In vivo study design and microsphere application

A prospective controlled experimental *in vivo* study was designed. Firstly, experimental OA was established in both rabbit joints (right and left) as explained above in chondrocyte isolation procedure. Microspheres (D MS and D-CS MS) were applied once by dispersing in 0.5 ml HA (Adant®, Tedej Meiji, Japan). After the establishment of OA (7th week after collagenase injections), the injections (HA, 5 mg D MS, 7.5 mg D MS, 5 mg D-CS MS, and 7.5 mg D-CS MS) were performed ($n = 5$ rabbits). The treatments and OA group were followed for another 8 weeks and the joints were then harvested for radiological, histological and hardness evaluations. The control group of healthy rabbits which were not given any medication was used for comparison.

Radiological examination

Anterior–posterior and lateral X-ray pictures of the knee joints were obtained using the Siemens Multix-C (Siemens AG, Erlangen, Germany) X-ray device as previously described.[25] Two blinded orthopedic surgeons independently scored the X-ray pictures according to Kellgren–Lawrence.[26]

Histological examinations

The joint specimens were immersed in 10% neutral formalin solution (pH 7.0) at room temperature. All specimens were decalcified in De Castro solution and embedded in paraffin. Sections (5–6 μm) were stained with hematoxylin/eosin (HE), Masson's trichrome (MT) and safranin O (SO). Photomicrographs of each sample were generated by a light microscope (Leica, DMR) attached computerized digital

camera (Model DFC 480, Leica Westlar). Both Mankin and the OARSI OA cartilage histopathology scoring systems were used.[27,28] All the samples were scored by two blinded observers and the averages were used.

Biomechanical analysis of cartilages

Hardness values of the medial and lateral femoral condyle were measured using a hand-held, self-contained material testing device (Kori Seiki, Japan) immediately after termination according to ASTM D2240 standards.[29] A scale from 0 to 99 was used in which low values represented softened cartilage. At each condyle three measurements were made and their average was calculated.

Statistical analysis

For *in vitro* studies, at each collection time One-Way ANOVA and Tukey's Multiple Comparison Tests among groups and within each groups, nonparametric Mann–Whitney U test were used (SPSS-15). For *in vivo* studies, the normality of distribution and the homogeneity of variances of the sample were checked using the Shapiro–Wilk test. Kruskal–Wallis (SPSS-15) was used for multiple comparison and Dunn (NCSS, 2007) as *post hoc* tests. Descriptive statistical values were expressed as median, minimum and maximum. Differences between groups were considered significant at $p < 0.05$ level.

RESULTS

The mean particle size of D MS was 75 μm and 90% of them were smaller than 152 μm, and 10% of them were smaller than 32 μm. The mean particle size of D-CS MS was 12 μm and 90% of them were smaller than 85 μm, and 10% of them were smaller than 4 μm. Doxycycline loading of D MS was 18% and D-CS MS was 10%, respectively. Doxycycline encapsulation efficiencies were 53 and 31% for D MS and D-CS MS, respectively. In addition, chondroitin sulfate loading of D-CS MS was 0.26% and encapsulation efficiency was 4%.

In vitro cytotoxicity studies using 3T3 fibroblast cells showed that the microspheres were biocompatible.[19] Proliferation of cells in agarose was also evident [Figure 2(A)] with increase in DNA amounts of all groups from day 9 to 15 and to 24. For *in vitro* bio-efficacy of the microspheres, other cell parameters were investigated. Among these, sGAG amount of the agarose constructs were found to be significantly higher in control group compared to IL-1β exposed groups at all time points [Figure 2(B)]. When compared pair-wise, the differences between IL-1β exposed groups were not statistically significant. Change in sGAG contents with respect to time showed significant increase from 9th and 24th day in all groups. Besides these, sGAG amounts released into the media were also measured. Control group had significantly lower amount of released sGAG than all other groups at 9th day [Figure 2(C)]. At the end of 15th and 24th days, however, only IL-1β exposed group was significantly higher than the other groups (control, IL-1β+D MS and IL-1β+D-CS MS). In Figure 2(D), hydroxyproline (HYP) content of 3D constructs are presented for the same incubation periods after being normalized to DNA amounts of each construct. On day 9, HYP contents of IL-1β and IL-1β+D MS groups had significant difference. On the other hand, HYP content of treatment groups and control group were not significantly different from each other at all time points. HYP contents of all IL-1β added groups showed increase from 9th to 15th and 24th days ($p < 0.05$). Pair-wise comparison of HYP contents of 15th and 24th days indicated that treatment groups were not statistically different from each other. When medium concentrations of HYP [Figure 2(E)] were measured, there were no significant differences between groups at 9th day. HYP concentration of media, indicating HYP degradation, significantly increased at 15th day. IL-1β+D MS group was statistically lower than the other 3 treatment groups. At 24th day, control versus IL-1β+D MS and IL-1β versus IL-1β+D-CS MS groups were not statistically significant.

As another important parameter of OA, MMP-13 concentrations were measured [Figure 2(F)]. Although IL-1β exposed groups and control group were not significantly different from each other at 9th day, numerically, IL-1β exposed group had the highest value. Yet, both IL-1β+MS treatments were observed to have MMP-13 amounts in between two groups (IL-1β group and control group). MMP-13 results of control and IL-1β group were significantly different from each other at 15th day [Figure 2(F)]. Also, both IL-1β+MS treatments decreased the MMP-13 amounts to the levels comparable with control. In the 24th day of the experiments, MMP-13 concentration of IL-1β treated groups and control group were again significantly different from each other. Both MS treatment groups were different from both control group and IL-1β group but unlike 15 day results these changes were not statistically significant. The D-CS MS microspheres had higher cumulative drug release than D-MS ones which was probably due to having smaller particle size or being more hydrophilic than D-MS microspheres (Figure 3). D-CS MS released ∼20 μg doxycycline; however, D MS released 15 μg doxycycline at the end of the 24 days. D-CS MS released ∼4 μg chondroitin sulfate at the end of the 24 days.

In vivo experiment design was summarized in Figure 4. The results were evaluated first with radiography. The radiology scores of the OA group had significantly ($p < 0.001$) inferior values when compared to the control group (Figure 5). Definite osteophytes with severe joint space narrowing and subchondral sclerosis confirmed the establishment of severe OA after intraarticular collagenase injection. Radiology score of the HA group was comparable to those of D MS (5 and 7.5 mg) groups. However, this score was significantly inferior to that of control ($p = 0.002$) and 5 mg D-CS MS ($p = 0.033$) treatment groups. Injection of D-CS MS improved the median radiological scores (5 mg: 1.5; 7.5 mg: 2.0) and were significantly lower than the OA group ($p = 0.001$ for 5 mg D-CS MS and $p = 0.022$ for 7.5 mg D-CS MS). Application of 5 mg D-CS MS displayed the closest value to the control group, which was significantly better than 5 and 7.5 mg D MS groups ($p = 0.025$, $p = 0.048$, respectively).

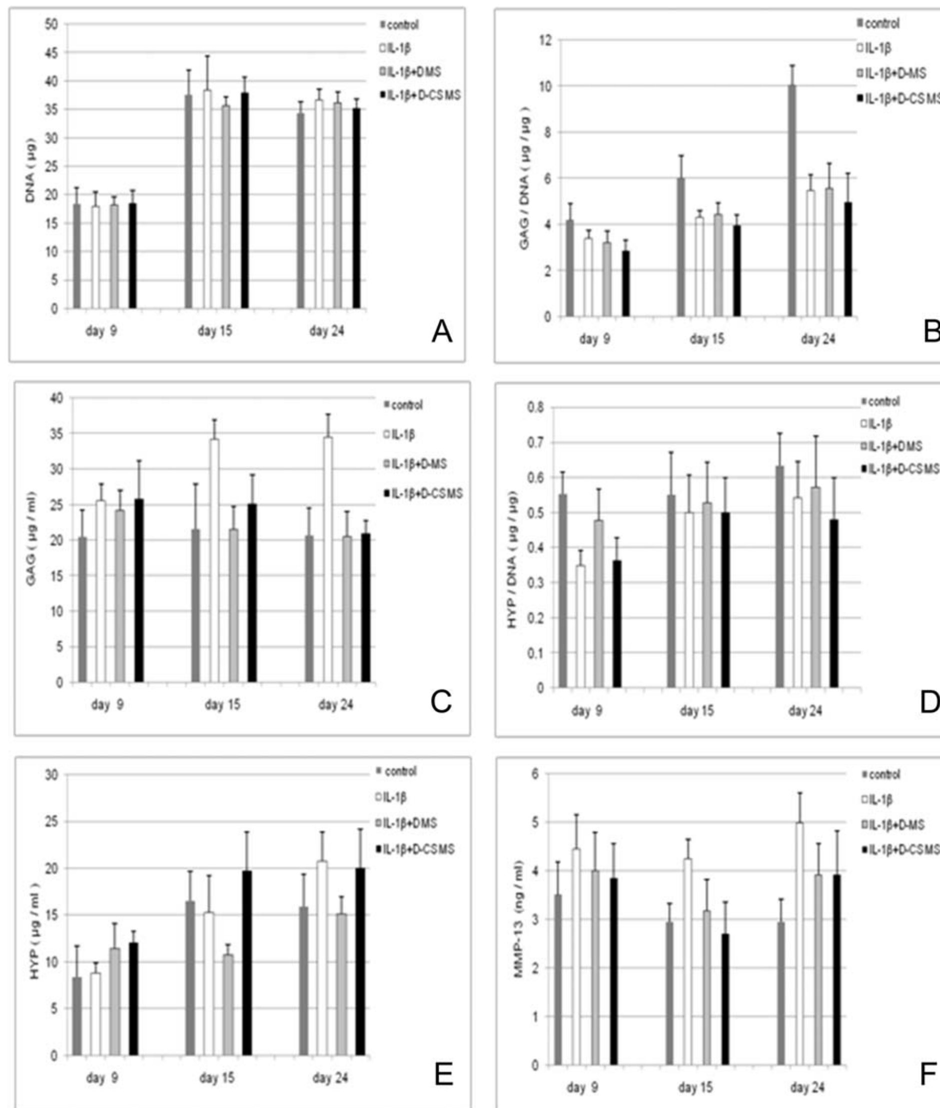


FIGURE 2. (A) DNA amounts, (B) GAG contents, (C) Hydroxyproline content of agarose-chondrocyte discs, (D) GAG amounts, and (E) Hydroxyproline amounts released into cell culture media, (F) MMP-13 levels in cell culture media ($n = 4$) at days 9, 15, and 24; Control: no-exposure to IL-1β; IL-1β: IL-1β added group; IL-1β+D MS: IL-1β added and treated with D loaded PCL microspheres; IL-1β+D-CS MS: IL-1β added and treated with D-CS co-loaded PCL microspheres.

In histological analysis results, OA group presented significantly inferior ($p < 0.001$) Mankin/Pritzker scores exhibiting grade 3 to 5 OA when compared to the control group which was also shown with microscopy images (Figures 6 and 7). Moderate to severe cartilage damage was noted in all the OA group rabbits that were partially restored at different levels in the treatment groups. However, none of the histological scores improved to the scores of the control group. The cartilage lesions consisted of the different degrees of the Safranin-O staining loss, surface fibrillation, vertical clefts, decreased number or hypertrophy/proliferation/degeneration of the chondrocytes. Cartilage degeneration was more prominent in the hyaluronan treated group when compared to the other treatment groups [Figure 6(A,B)]. D MS (7.5 mg) group (Mankin $p = 0.007$, Pritzker $p = 0.023$) and both doses of D-CS MS (5 mg-Mankin $p < 0.001$, Pritzker $p = 0.001$ and 7.5 mg-Mankin $p < 0.001$, Pritzker $p < 0.001$) treatments significantly improved the Mankin/Pritzker scores when compared to those of the OA group. D-CS MS group presented superior scores compared to the HA group with both of D-CS MS treatment doses (5 mg-Mankin $p = 0.036$, Pritzker $p = 0.038$ and 7.5 mg-Mankin $p = 0.013$, Pritzker $p = 0.011$). The D MS and the D-CS MS groups exhibited similar histological scores showing comparable therapeutic performance. The only exception was the significantly lower Mankin and Pritzker scores (indicating better treatment potency) of the 7.5 mg D-CS MS group compared to that of the 5 mg D-MS group (Mankin $p = 0.048$ and Pritzker $p = 0.033$) [Figure 7(A,B)].

Biomechanical evaluations (hardness) of the lateral and medial femoral condyles of the control group were higher than the other groups except the D-CS MS groups (Figure 8).

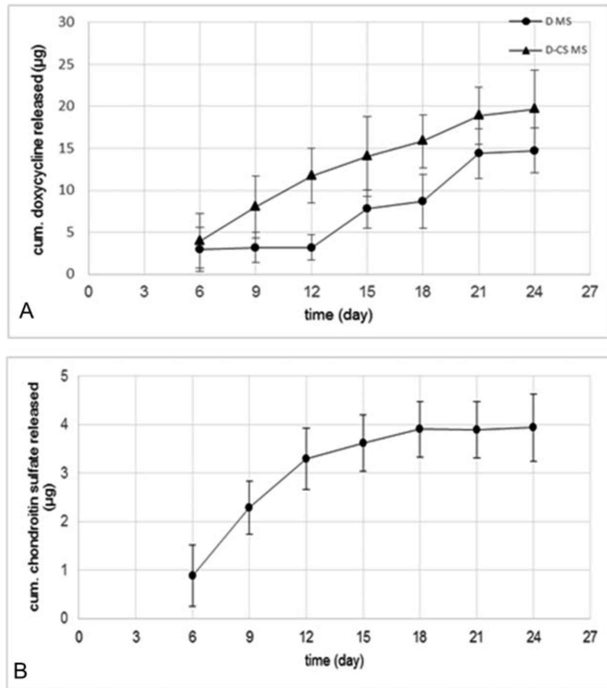


FIGURE 3. Release profiles of (A) doxycycline, (B) chondroitin sulfate from microspheres in cell culture media ($n = 4$).

Among treatments, 5 mg D-CS MS group had the highest hardness values (75.0 for lateral and 72.2 for medial femoral condyle), which were not significantly different than the control group. For lateral femoral condyle, the hardness of the control cartilage was significantly higher than that of the 5 mg D MS (51.8; $p = 0.026$) group. Hardness value of lateral femoral condyle of 5 mg D-CS MS group was significantly higher than that of OA, HA, and both of the D MS groups

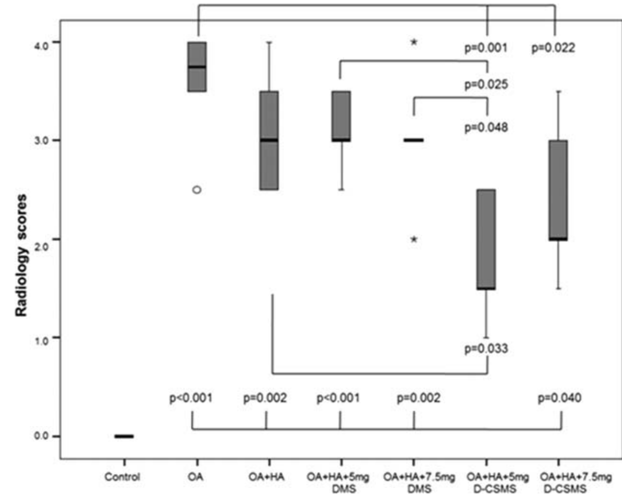


FIGURE 5. Radiology scores of rabbit knee joints.

($p \leq 0.015$). Although 7.5 mg D-CS MS treatment also had high numerical value (68.2), it showed significant difference only from HA ($p = 0.030$) and, 5 mg D MS ($p = 0.002$) treatments [Figure 8(A)]. Hardness outcomes of the medial femoral condyle showed that the control group had highest value except D-CS MS (5 mg: 72.2; 7.5 mg: 67.3) treatments. However, for the medial femoral condyle, hardness of the control group was not statistically different from any other group. Considering the medial femoral condyle, OA group was significantly lower than both D-CS MS treatment doses ($p < 0.001$ for 5 mg and $p = 0.018$ for 7.5 mg). 5 mg D MS group (48.8) was significantly lower than both D-CS MS treatment groups ($p < 0.001$ for 5 mg and $p = 0.005$ for 7.5 mg). Furthermore, 7.5 mg D MS (53.5) group had significantly lower hardness

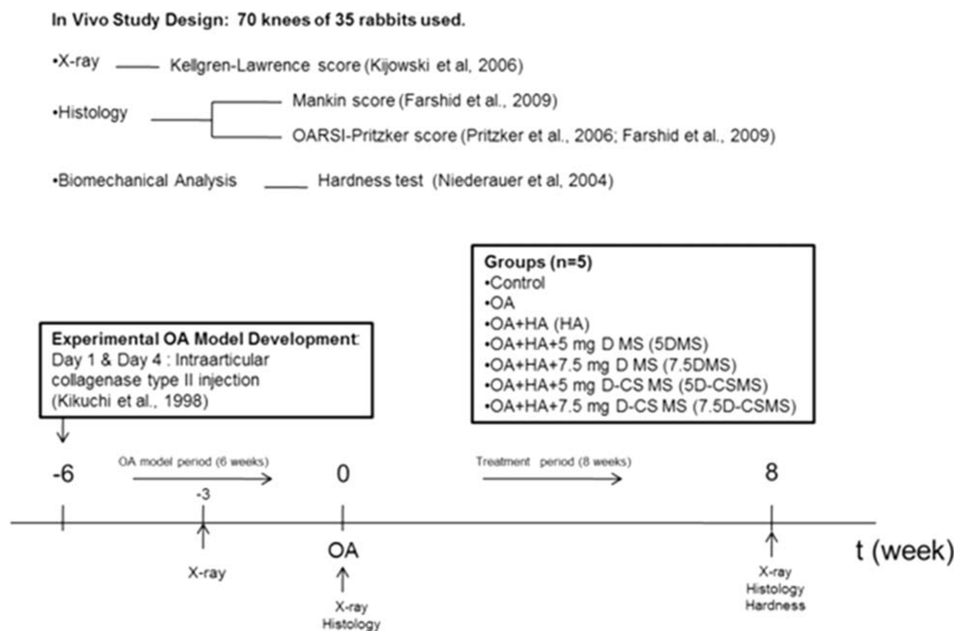
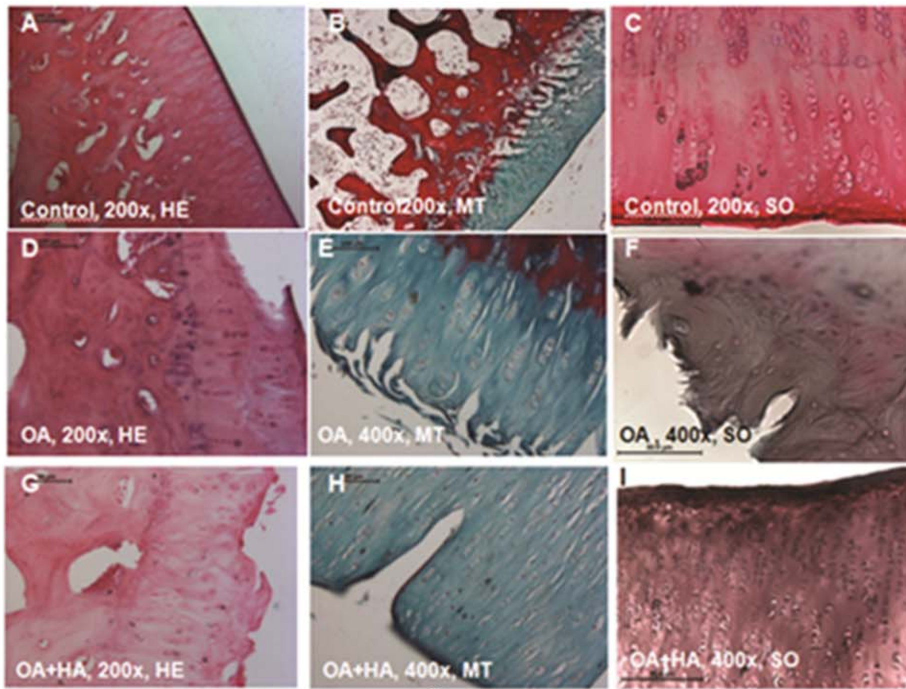


FIGURE 4. Outline of the *in vivo* study. About 6 weeks after OA induction, rabbits were divided into seven independent groups and the related treatment was applied. About 8 weeks after treatment, rabbits were evaluated according to dependent variables.



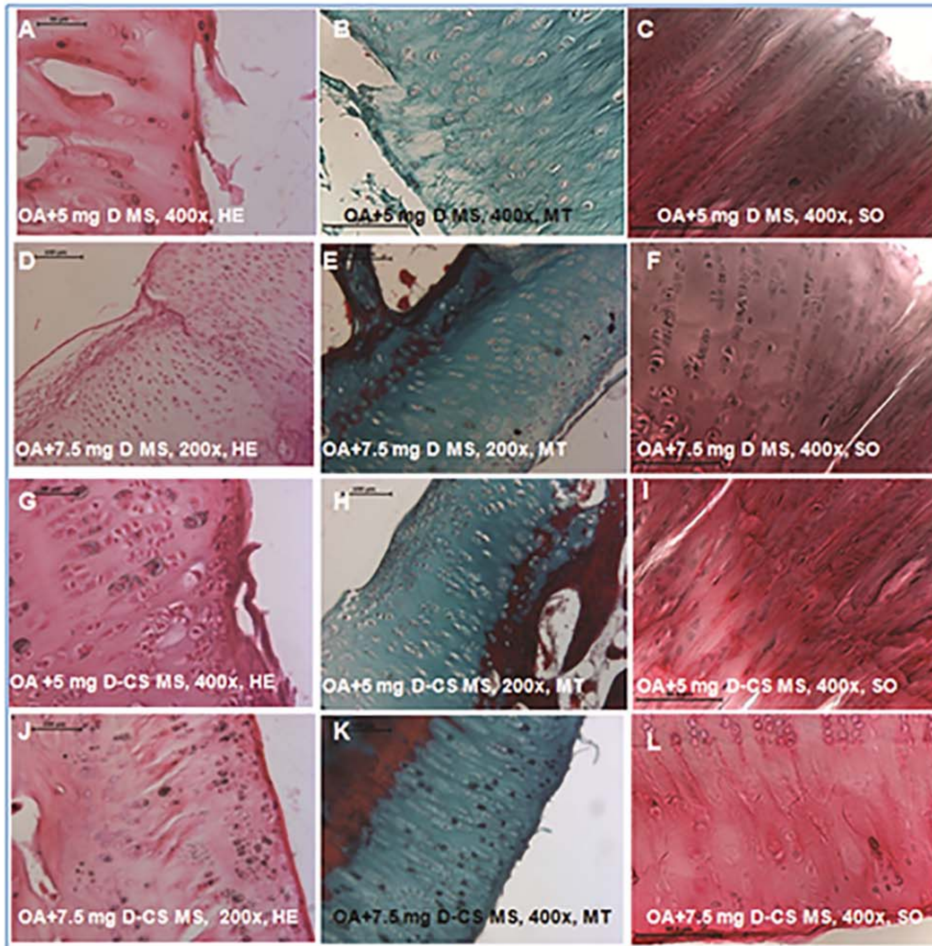


FIGURE 6. (a) The articular cartilage of the control joints exhibited normal morphology with smooth cartilage surface, intact tidemark integrity and healthy chondrocyte organization. In D-I, different grades of OA-induced cartilage degeneration with fibrillation, cleft formation (in H), chondrocyte damage and matrix degeneration, loss of safranin O staining (in F and I) are observed. Bone is exposed in F. The degeneration severity in the HA groups (G-I) is less than the OA group. HE: Haematoxylin eosin, MT: Masson's trichrome, SO: Safranin O. (b) The articular cartilage of the different treatment groups exhibits mild to moderate OA-induced degeneration with fibrillation, chondrocyte damage and matrix degeneration. The degeneration severity is less than that of the OA group in all groups. Note that the safranin O staining is regained to some extent with the treatment modalities (in C F, I, and L). In J to L the mildly rough to smooth articular surface is apparent. HE: Haematoxylin eosin, MT: Masson's trichrome, SO: Safranin O. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

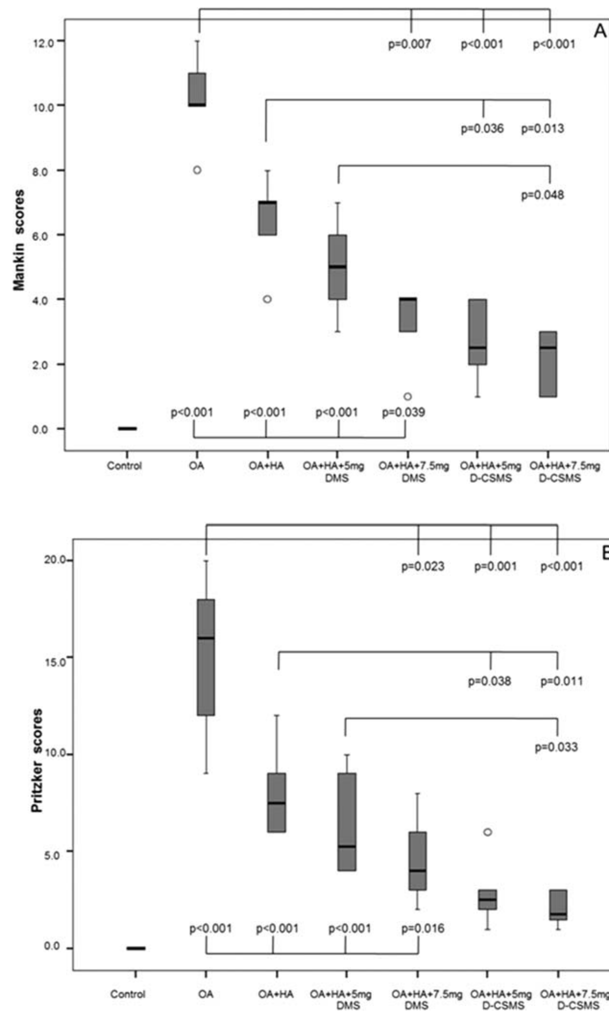


FIGURE 7. Descriptive statistical data of the (A) Mankin scores and (B) OARSI-Pritzker scores for *in vivo* study.

values than both of the D-CS MS ($p = 0.003$ for 5 mg and $p = 0.038$ for 7.5 mg) treatment groups [Figure 8(B)].

DISCUSSION

Three-dimensional agarose gel culture model has been used widely because this model allows induction of inflammation and degradation of cell cultures by cytokines such as IL-1β without changing native chondrocyte phenotype in culture.[22] It was reported that agarose was ideal for chondrocytes since it enables articular chondrocytes to remain viable, to differentiate and produce ECM.[22] To mimic the diseased synovial fluid and keep the diseased state, IL-1β,[30] was added to the media.[22,31] The decrease in sGAG content of agarose constructs upon IL-1β application was thought to be caused by reduced synthesis or increased degradation rate or both.[22] Therefore IL-1β added groups had lower GAG content than control group at 9th, 15th, and 24th days as expected. Yet, in these groups, GAG contents of the agarose-chondrocyte constructs increased after 15 days of incubation upon proliferation of chondrocytes. The fact that IL-1β addition to the media caused suppression of the GAG production was reported by histochemical and biochemical studies.[22] It is also known that when degradation of sGAG molecules increases, they can be released into surrounding environment from the matrix. At 15th and 24th days of incubation, released GAG was higher in IL-1β group than D MS and D-CS MS treatment and control groups indicating the prevention of GAG degradation with released doxycycline. *In vitro* and *in vivo* studies reported inhibitory effect of tetracycline group antibiotics on collagen degradation by MMPs, and thereby, their potential of therapeutic use in OA. During OA development, cytokines such as IL-1 produced by activated synoviocytes, mononuclear cells or by cartilage itself significantly up-regulate MMPs.[32] Cartilage is subjected to proinflammatory cytokines during OA and first aggrecanase activity occurs. GAG release at the very beginning of degradation may also interceded by other enzymes unaffected by doxycycline such as hyaluronidases or aggrecanases.[16] Aggrecanase enzymes are responsible for aggrecan catabolism which is stimulated with IL-1.[22] In our study due to use of osteoarthritic rabbit chondrocytes in agarose

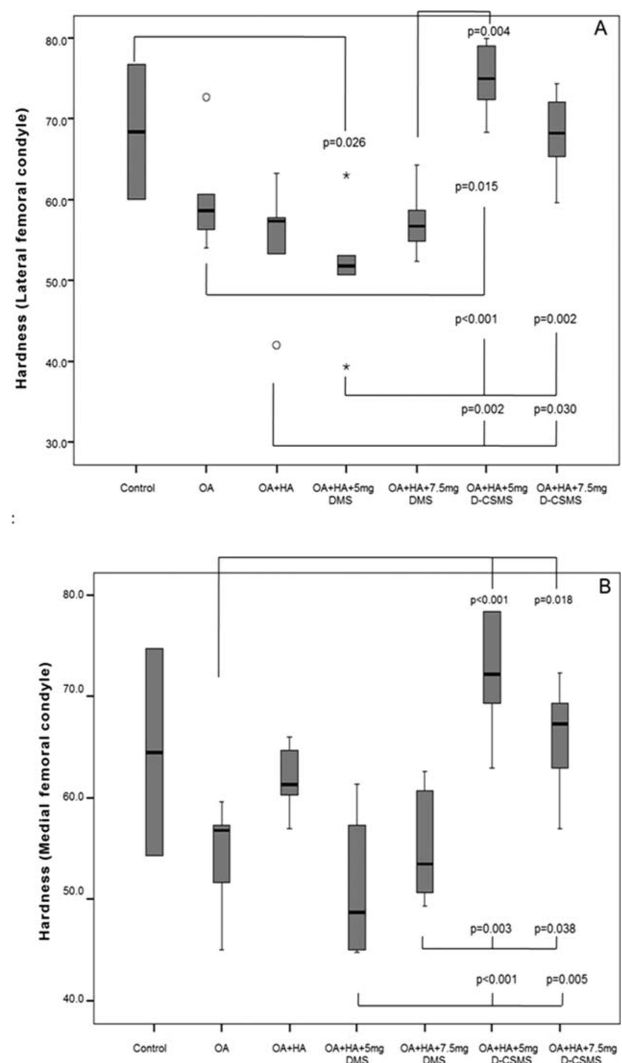


FIGURE 8. Hardness measurement results of rabbit knee joints for (A) the lateral femoral condyle and (B) the medial femoral condyle.

culture, GAG loss due to other enzymes may have been started during monolayer culturing. At the 9th day the GAG loss cannot be stopped since doxycycline level may not be enough to inhibit the degradation process. However, at the 24th day, increased doxycycline level (upon release from microspheres) inhibited MMP-13 followed by a decrease in GAG loss. IL-1β is known to upregulate MMP-13 production.[32,33] The early treatment with doxycycline may have beneficial outcomes in the long-term.[16] However, in late stage of cartilage degradation, increase in level of IL-1 may result in some low levels of primary MMP-generated catabolism of aggrecan.[34] At the 24th day MMP-13 level of MS treated groups did not decrease to the control level. This may be due to insufficiency of released amount of doxycycline or owing to MMP-generated catabolism of aggrecan. Here, doxycycline release inhibited the MMP-13 activity, but to different degrees at different time points. While MS treatments were not significantly different from each other at any of the time points for both groups, the inhibition level of MMP-13 decreased between 20 and 24th days of the *in vitro* culture. This might be suggested to be owing to wash-out of the released drug during cell culture media changes. The numerical decrease in HYP content of the agarose constructs of theIL-1β added groups compared to control group at day 9 indicated that IL-1β had negative effect on collagen metabolism but this effect was not clearly observed as in the sGAG case. Enzymes that degrade proteoglycans are "aggrecanases." Nevertheless, it was reported that aggrecan degradation was not dependent on this MMP activity.[31] IL-1β alone was not adequate to degrade collagens. It was reported that this requires other factors such as presence of several enzymes found in synovial fluid of arthritis patients. At the 24th day, D MS and D-CS MS treatment decreased the MMP levels. Doxycycline acts directly on collagenase to inhibit its activity and indirectly to reduce collagenase levels.[35] The loading efficiency of CS was lower than the expected. However, due to bulky structure of CS besides its high negative charge and hydrophilic properties it was not possible to load this molecule at higher percentages. Even it was not possible to obtain CS loaded microspheres at higher initial loadings. Consequently, CS co-loading to MS results with swelling of the polymeric matrix and causes increase in release rate of doxycycline from microspheres. It was reported that 2.8 U activity of MMP-13 against collagen type II was inhibited up to 50–60% by 30 μ*M* doxycycline application.[36] Although D-CS MS released doxycycline higher than D MS, the decrease in MMP-13 content of these two groups was not significantly different from each other. The main reason of this might be the doxycycline amounts being not very high and it was replenished with each medium exchange.

According to radiological evaluations, OA group had significantly inferior values than control group indicating *in vivo* establishment of OA. Radiology outcomes showed positive effects of 5 and 7.5 mg D-CS MS on OA with superior scores compared to OA, HA, D MS groups. Plain radiography is still the first and most important imaging technique for evaluation of a known or suspected diagnosis of OA. Among the several grading schemes developed, the most widely utilized is Kellgren–Lawrence grade (KLG) classification.[26,37] Oral doxycycline treatment given to patients with unilateral OA for 30 months significantly decreased the rate of joint space narrowing.[16] Our histological analysis results also supported the radiology outcomes, where treatment with both D MS and D-CS MS groups improved the recovery from OA compared to HA and OA groups. In parallel with radiology results, all microsphere treatment groups exhibited similar therapeutic performance. Among treatments significantly lower Mankin and Pritzker scores were obtained with the 7.5 mg D-CS MS group compared to that of the 5 mg D MS group showing that the most efficient treatment is achieved with this composition and amount.

Owing to its degenerated matrix, osteoarthritic cartilage is weaker (has less stiffness) than the normal cartilage in terms of mechanical properties. Low hardness values are considered as the sign of the softness of materials. Niederauer et al.[29] suggested that change in articular cartilage stiffness detected by indentation probe is a good indicator of cartilage damage. The probe readings as well as the aggregate modulus, correlate strongly with artificially induced cartilage damage.[29] In our study, hardness measurements obtained by indentation of cartilages demonstrated decreased values in the OA group compared to control group but this difference was not significant. Improvement in hardness property was most evident with highest values of D-CS MS groups among treatments. The hardness results of D-CS MS treatments were comparable to control group and it was in accordance with the other analyses' outcomes. However, hardness values of HA and D MS treatments were not different than OA group. Proteoglycans are considered as one of the major structural components of cartilage and decrease in its amount results in lower mechanical strength, or stiffness of the cartilage[29,33] whereas degradation and loss of type II collagen by collagenases (MMPs-1 and −13) result in an irreversible loss of tensile properties and structural integrity.[38] Thus, osteoarthritis makes the cartilage tissue less resistant to indentation, or softer compared to the healthy state.[27,39] The values for young and normal human cartilage samples (73.0 for the lateral and 64.0 for the medial femoral condyle) may be used as reference (normal) values against which other values measured by the probe can be compared.[39] In our study, healthy cartilages had values app. 68.3 and 64.5 for the lateral and medial femoral condyle measurements, respectively. Bae et al. also studied healthy and degenerated cartilage of different age groups of human cadaveric donors by indentation testing performed at the medial and lateral femoral condyles of articular cartilage.[39] They observed that at any age, degenerated cartilage had significantly lower indentation stiffness than healthy cartilage (30–37 vs. 65–68).

In conclusion, advantages of controlled drug delivery systems over conventional drug therapies can be used to overcome limitations and problems in osteoarthritis treatment and for enhanced treatment success. Developed controlled release system of doxycycline and Chondroitin sulfate showed this potential for local treatment of OA when applied by intraarticular injection within HA. *In vitro* characterization

studies showed enhancement of release of doxycycline by co-loading with a hydrophilic molecule, CS. However, due to difficulty of encapsulating CS at higher amounts effects of released chondroitin sulfate was not very high compared to only doxycycline loaded microspheres. *In vitro* and *in vivo* results were strongly suggesting the potential of D MS and D-CS MS for recovery from the detrimental effects of OA. In overall evaluations D CS-MS provided most efficient treatment outcomes. Increasing chondroitin sulfate loading is suggested for enhancement of its potency.

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