RESEARCH ARTICLE

Combination of collagen scaffold with doxycycline for the treatment of cartilage and subchondral bone defects

Authors
Haixiang Liang[1], Spencer Stein[2], Pooja Swami[1], Henintsoa Fanjaniaina A.[1], Daniel Grande[1*]

Affiliations

[1] Orthopedic Research Laboratory, Feinstein Institutes for Medical Research, Northwell Health, Manhasset, New York, USA.
[2] Department of Orthopedic Surgery, Long Island Jewish Medical Center, New Hyde Park, New York, USA.

***: Corresponding author:**
Daniel A. Grande, Ph.D., Orthopedic Research Laboratory, Feinstein Institutes for Medical Research, Northwell Health, 350 Community Dr, Manhasset, New York, 11030, USA. Email: dgrande@northwell.edu

Abstract
The repair of cartilage and bone tissue post arthritis or injury in the knee joint is a challenge to the orthopedics. Both doxycycline and collagen scaffolds had been shown with positive influences on the repair of the tissue. Doxycycline inhibits catabolism related enzymes. Collagens provide tissue repair materials. In this study, the influence of doxycycline and jellyfish collagen (JFC) scaffold to the cells in vitro and tissue repair in vivo was studied. Doxycycline was found with a broad inhibition of the matrix metallopeptidases. It also had an enhancement to the progenitor chondrocytes but suppressed the maturation of chondrocytes. JFC (major type II collagen) was seen had improved both type I and type II collagen production during chondrogenesis of mesenchymal stem cells in vitro. Both doxycycline and JFC enhanced the bone formation and had synergistic effects in a late-stage during the bone repair process. However, the cartilage repair was found with no significant influence after the implantations. Overall, the control of metabolism by doxycycline and providing stimulation to collagen production by the implantation of JFC only contribute to bone growth but had little influence on cartilage repair. This indicates the growth of cartilage tissue needs more delicate regulation. The subchondral bone repair, even with some chondrocytes and proteoglycan deposition in the extracellular matrix could not convert to a repair with mature hyaluronic cartilage tissue.

Keywords: Doxycycline; Jellyfish collagen; type II collagen; matrix metallopeptidases; bone defect; cartilage defect.

1. Introduction

Addressing the limited healing capacity of articular cartilage alongside the pathological alterations in the underlying subchondral bone compartment is a major challenge in the development of therapeutic strategies for the treatment of arthritis or other diseases of the joint. In order to rebuild cartilage and bone tissue, it is essential to regulate the metabolism of these cells to promote tissue reconstruction and ECM synthesis. Doxycycline is an antibiotic, known to inhibit the activities of matrix degradative enzymes like matrix metalloproteinases (MMPs) and thus improve tissue repair.[1,2] Matrix degradation and prolonged inflammation are hallmarks of disease progression in arthritis and other diseases of the joint. Hence, inhibition of MMPs by doxycycline helps slow the development of arthritis.[3] Doxycycline has been previously reported to promote bone healing.[4,5] Furthermore, the combination of doxycycline with other biological factors has been tested for application in tissue engineering including the repair of musculoskeletal tissues.[1,6] Collagen, a major protein content of the musculoskeletal system, is a backbone of cartilage and bone tissue repair. Marine collagen has been considered as an alternative to vertebral collagen for fabricating biological scaffolds.[7,8] Jellyfish is one of the major sources of marine collagen.[9] Different from the widely available vertebral collagen, which is made with collagen type I or III, Jellyfish collagen (JFC) is mainly composed of collagen that closely resembles collagen type II in vertebrates.[10] In this study, we tested the combinatory application of doxycycline and JFC scaffold in the repair of cartilage and bone defects in the knee joints.

2. Methods

2.1 Cell isolation and culture

Human chondrocytes were collected from discarded tissues from 2 adult donors undergoing knee joint surgery unrelated to infection or arthritis. The cartilage tissues from the donors were collected and immediately put into a sterile container with saline to be processed for cell isolation. After being washed in phosphate buffered saline (PBS) with 2% antibiotic-antimycotic (ThermoFisher Scientific, Grand Island, NY) 4 times, the harvested cartilage tissue was treated with 0.05% hyaluronidase (MP Biomedicals, Irvine, CA) in PBS for 10 minutes, rinsed with PBS again, and then cut into small pieces using a surgical blade. The resulting cartilage fragments were subsequently treated with 0.2% Trypsin solution and placed in a water bath at 37°C for 10 minutes with continuous shaking. Following 3 washes with PBS and brief centrifugations, the cartilage tissues were digested in 0.4% collagenase type II (ThermoFisher Scientific, Grand Island, NY) in Dulbecco's Modified Eagle Medium (DMEM)/F12 medium, and placed in the water bath at 37°C for 90 minutes with agitation. The supernatant was then collected, and centrifuged at 1000 rpm for 10 minutes to obtain a cell pellet. All pellets were collected and cultured in monolayer in DMEM/F12 medium with 10% fetal bovine serum (FBS) and 1% anti-anti at 37°C with 5% CO_2 in the incubator. Cells at passage 3-5 were used for this study.

Human bone marrow mesenchymal stem cells (MSC) were purchased from ATCC (American Type Culture Collection, Manassas, VA). Cells were cultured in DMEM/F12 medium containing 10% FBS and 1% anti-anti and the medium was changed every 3 days. The MSCs used in this study were at passage 5.

2.2 In vitro doxycycline treatment

Human chondrocytes were seeded in 12-well cell culture plates with 3×10^5 cells per well. After the cells reached 95% confluency, they were treated with doxycycline (Sigma-Aldrich, St. Louis, MO) at varying concentrations with respect to their groups. The cells were separated into 3 groups: a low dose group with doxycycline at 2µg/mL, a high dose group with doxycycline at 20µg/mL, and a control group without doxycycline. The treatments were given every 2 days. All groups were harvested at 4 and 8 days after initial treatment.

2.3 MSC Cells cultured with collagen scaffold in vitro

Every 100mg type II collagen scaffold made from jellyfish *Rhopilema esculentum* (JFC) (Agam Group, Rehovot, Israel) was put into 2.5mL medium (DMEM/F12 + 10% FBS), then cut in gentleMACS C tubes (Miltenyi Biotec, Bergisch Gladbach, Germany) for 14 minutes. The scaffold particles were then mixed with cell suspension in a similar medium to make a mixture containing a final cell concentration of 1.5×10^5 cells and 4.5mg collagen particles per milliliter of medium. Then every 2mL mixture was centrifuged at 500g for 10 minutes to make a cell pellet. A cell pellet with a similar cell concentration of human MSCs but without the mixture of collagen particles was used as a control group. Both groups were cultured in DMEM/F12 containing 1% FBS, 1% Insulin-transferrin-selenite (ITS) (ThermoFisher Scientific, Grand Island, NY), 200uM sodium ascorbic phosphate (Sigma-Aldrich, St. Louis, MO), 10nM dexamethasone (Sigma-Aldrich, St. Louis, MO), and 100ng/mL growth and differentiation factor 5 (GDF5) (ProSpec-Tany TechnoGene, Ness-Ziona, Israel). Medium was changed every 3 days. The samples were collected at 1- and 2-week time points for gene expression study.

2.4 Alcian blue staining

Both groups of MSC pellet culture had samples collected at a 2-week time point for histological evaluation with alcian blue staining following the standard protocol. Briefly, the cell pellets were fixed in 10% formalin (ThermoFisher Scientific, Grand Island, NY) for 15 minutes. Then the pellets were washed in PBS for 3 times. After being embedded in paraffin (ThermoFisher Scientific, Grand Island, NY), the pellets were sectioned into 5µm thin slices which were placed on slides. The slides were stained in alcian blue solution (pH 1.0) (ThermoFisher Scientific, Grand Island, NY) for 5 minutes.

2.5 Gene expression studies

Human cells (including chondrocytes and MSCs) collected either from monolayer or pellet cultures were used for gene expression study to detect biomarkers. Messenger RNA from cells was collected and purified using RNeasy mini kit (QIAGEN, Venlo, Netherlands). The reverse transcription of mRNA to cDNA was performed by using a cDNA Synthesis Kit and a T100 Thermal Cycler (Bio-Rad Laboratories, Inc., Hercules, California). The expressions of target genes were measured by Realtime PCR with LightCycler 480 (Roche, Basel, Switzerland) and iQ SYBR Green Supermix (Bio-Rad) following standard protocols. The target genes in this study included SRY-Box Transcription Factor 9 (Sox9), Type I collagen, Type II collagen, Aggrecan, metalloproteinase-1 (MMP1), MMP3, MMP9, MMP13, Discoidin Domain Receptor Tyrosine Kinase 2 (DDR2), Runt-related transcription factor 2 (RunX2), and Alkaline Phosphatase (ALP). The expression of each target gene was normalized by using GAPDH as reference genes and compared among groups by using 2ˆ(–delta delta CT)

method. (The sequences of the primers was listed in Table 1)

Table 1. Primer sequences

Primer	Sequences
GAPDH	ACCCAGAAGACTGTGGATGG GAGGCAGGGATGATGTTCTG
MMP1	TGGTGTCTCACAGCTTCCCA CTCCACATCTGGGCTGCTTC
MMP3	ATCCTACTGTTGCTGTGCGT CTTCCCCGTCACCTCCAATC
MMP9	CCCGGAGTGAGTTGAACCA CAGGACGGGAGCCCTAGTC
MMP13	GGACAAGTAGTTCCAAAGGCTACAA CTTTTGCCGGTGTAGGTGTAGATAG
Sox9	ACCACCCGGATTACAAGTACCA TTGAAGATGGCGTTGGGGGAG
Type I collagen	TGACCTCAAGATGTGCCACT ACCAGACATGCCTCTTGTCC
Type II collagen	CCCAGAGGTGACAAAGGAGA CACCTTGGTCTCCAGAAGGA
Aggrecan	GGCACTAGTCAACCCTTTGG CTGAACCCTGGTAACCCTGA
RunX2	ATGCTTCATTCGCCTCAC GATGCCATAGTCCCTCC
ALP	TTGCTGGTGGAAGGAGGC GTGAAGACGTGGGAATGG
DDR2	GGTTGGGGAAACGCAGTGG GGTCCTGGGAGGCATATCAA

2.6 In vivo implantation

The animal work in this study was approved by the Institutional Animal Care and Use Committee (IACUC) of Feinstein Institutes for Medical Research. A total of 16 male Sprague-Dawley (SD) rats (Charles River, Wilmington, MA) weighing approximately 350g were used for in vivo study. Each rat received an operative cartilage and subchondral bone defect created on both knee joints following methods previously published.[11] Briefly, the rat receiving the surgery was anesthetized by isoflurane. The skin was prepared by using aseptic techniques. The knee joint was exposed following an incision along the medial edge of the patella tendon. The patellar tendon was pushed to the lateral side to expose the surface of the distal end of the femur. A defect was created on the trochlear groove by using a 2mm diameter ball shape burr drill bit, which was about 2mm in depth to reach the subchondral bone area. The defect on one leg was randomly chosen to receive an implantation of JFC scaffold, while the contralateral side received no implantation. The incision was closed layer by layer with 4-0 sutures. Post operation, half of the rats received 1% doxycycline in water by oral gavage with 10mg/kg per day post-surgery. The animals were euthanized at 4-and 8-weeks post-surgery and the femur samples were used for histological studies.

2.7 Histological study of in vivo experiment

The femur samples of the rats were collected at each time point. The samples were fixed in 10% formalin (ThermoFisher Scientific, Grand Island, NY) for 1 week. Then the samples were decalcified with 0.25M Ethylenediaminetetraacetic acid (EDTA) (ThermoFisher Scientific, Grand Island, NY) in PBS (pH 7.4) on the shaker for 2 weeks. After being embedded in paraffin (ThermoFisher Scientific, Grand Island, NY), samples were sectioned into 7μm thick slices and were then stained in Safranin O/fast green staining.

2.8 Statistical analyses

The results of gene expression study were expressed as mean ± standard deviation (SD). The expression of each gene was compared among groups by using One-way ANOVA analyses. Significance was determined if $p < 0.05$.

3. Results

3.1 Human chondrocytes treated by doxycycline

Monolayer cultured human chondrocytes were treated with low dose (LDD) and high dose (HDD) of doxycycline for 4 and 8 days. The expressions of MMP1, MMP3, MMP9, and MM13 were evaluated. At the 4-day time point, the expressions of MMP1 and MMP13 had no significant change among groups. The LDD group had a lower level of MMP3 and MMP9 than the control group. The HDD group showed only MMP3 expression to be significantly lower than the control group at the 4-day time point. However, in the 8-day time point, the HDD group had much lower expressions of MMP1, MMP3, and MMP13 than other two groups. But the expression of MMP9 had no significant difference among groups. (Figure 1 A, B, C, D)

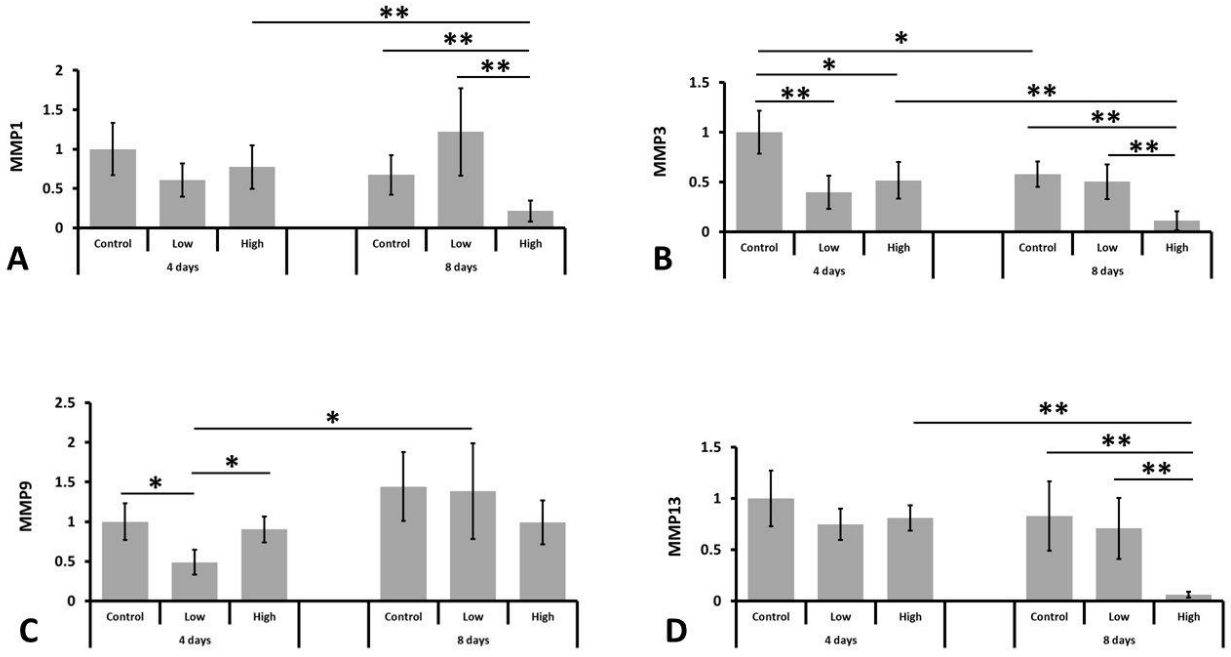

Figure 1. Comparison of relative gene expression of MMP1, MMP3, MMP9 and MMP13 in human chondrocytes among groups. The results are shown as relative fold change. *: p<0.05; **:p < 0.01. (A) MMP1; (B) MMP3; (C) MMP9; (D) MMP13.

The expressions of Sox9 and ALP had no significant differences among groups at day 4. However, the expression of Collagen type II had a lowest level in the HDD group. In contrast, the HDD group had the highest level of expression of RunX2 among three groups. At day 8, the HDD group had the lowest level of Collagen type II, RunX2, and ALP compared to the other two groups. But both LDD and HDD groups had higher levels of Sox9 than the control group. (Figure 2 A, B, C, D)

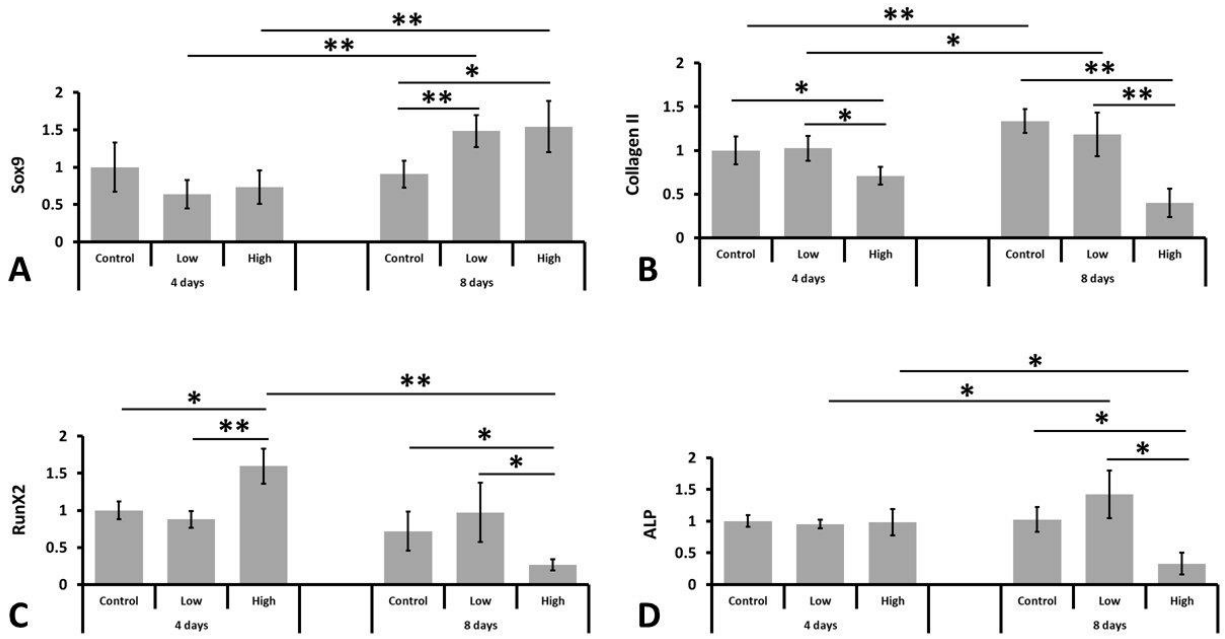

Figure 2. Comparison of relative gene expression of Sox9, Type II collagen, RunX2 and ALP in human chondrocytes among groups. The results are shown as relative fold change. *: $p<0.05$; **:$p < 0.01$. (A) Sox9; (B) Type II collagen; (C) RunX2; (D) ALP.

3.2 Human MSC pellet culture with scaffold particles

Human MSCs were pellet cultured with or without the collagen type II particles. Gene expression was compared among groups at 1- and 2-week time points. There was not a significant difference between groups in the expression of Sox9 at both time points. However, the expression of collagen type II significantly increased in the experimental group than the control at 2-week time point. The experimental group also had a higher level of aggrecan than the control at the 1-week time point. Both groups had an increased expression of collagen type I at the 2-week than the 1-week time point. Moreover, the experimental group had a higher level of collagen type I expression than the control group at both time points. For the expression of MMP13, the experimental group had a higher level at the 1-week time point. But it was significantly lower than the control group at the 2-week time point. The difference was majorly caused by a significant increase of MMP13 expression in the control group between two time points. Similar situation was also seen in the expression of DDR2. There was a significant increase in the expression of DDR2 in the control group, the experimental group had a much lower level of DDR2 at the 2-week

time point. (Figure 3 A, B, C, D, E, F) Histological study showed both groups had positively stained green color after culture in the chondrogenic induction medium for 2 weeks. The control group had a more condensed pellet. But the experimental group formed a loosen pellet due to the mixture with the JFC collagen particles. (Figure 4 A, B, C, D)

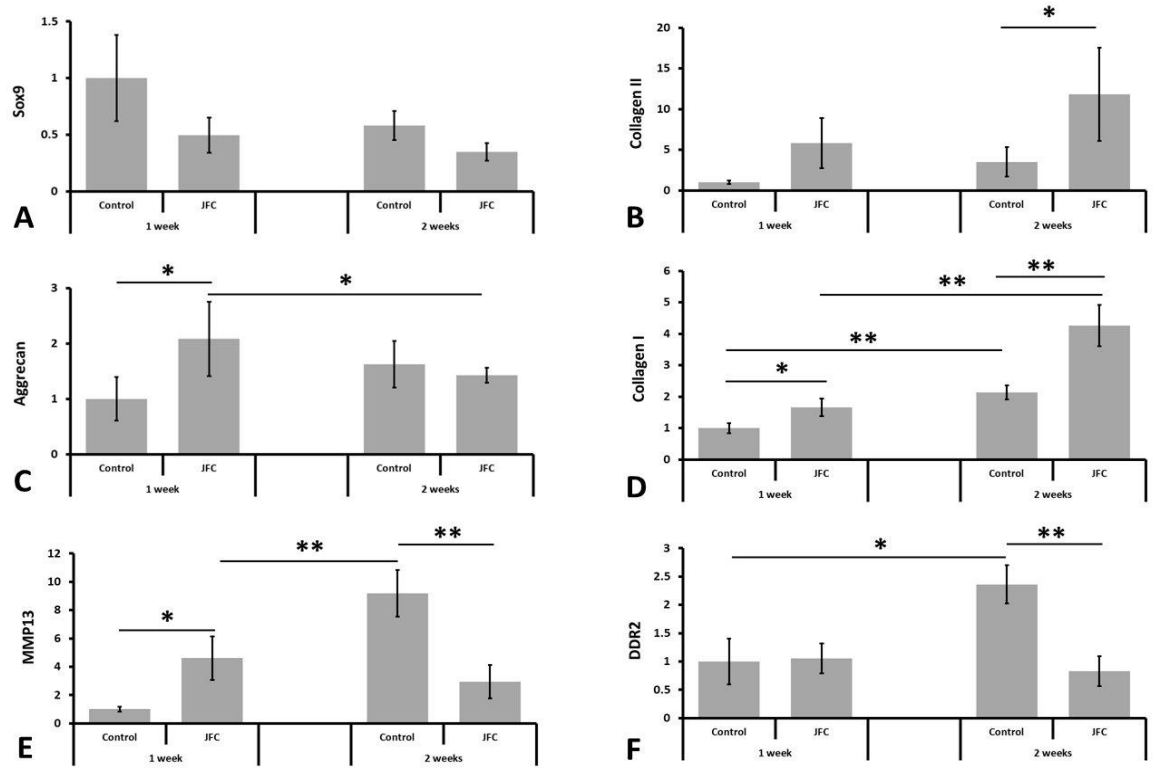

Figure 3. Comparison of relative gene expression of Sox9, Type II collagen, Aggrecan, Type I collagen, MMP13 and DDR2 in pellet cultured human MSC among groups. The results are shown as relative fold change. *: $p<0.05$; **:$p < 0.01$. (A) Sox9; (B) Type II collagen; (C) Aggrecan; (D) Type I collagen; (E) MMP13; (F) DDR2.

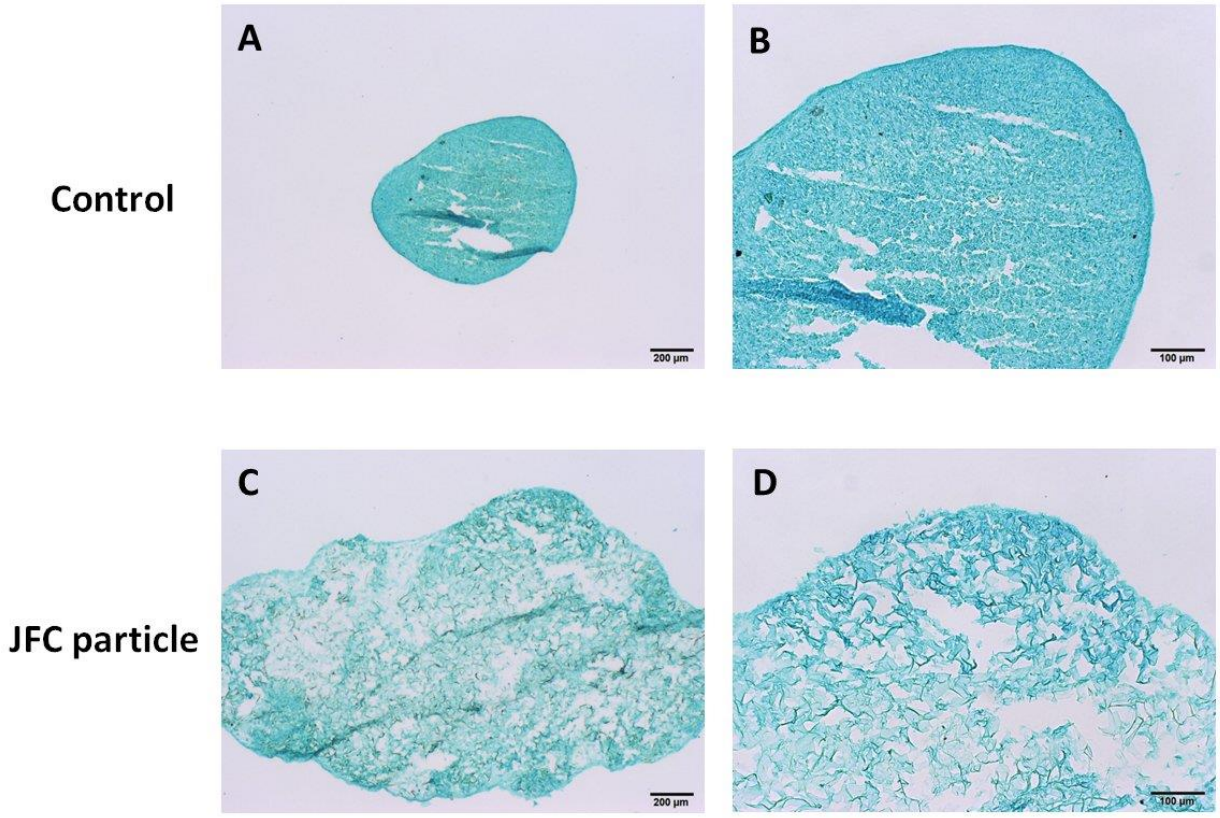

Figure 4. Alcian Blue staining of pellet cultured human MSC treated with chondrogenic induction medium for 2 weeks. (A) Control group low magnification. (B) Control group high magnification. (C) Mixture with JFC particles low magnification; (D) Mixture with JFC particles high magnification.

3.3 In vivo implantations

The in vivo implantation of JFC scaffolds treated with or without doxycycline was evaluated at 4-and 8- weeks. At the 4-week time point, the control group without scaffold had a fibrous tissue covering the surface of the defect. Some bone tissue was seen in the defect with a large hollow space filled by bone marrow tissues. (Figure 5A) In the JFC scaffold only group, the defect contained a mixture of cells, fibrous and bone tissues with fragments of degraded scaffolds. On the edge of the defect, cartilage was seen growing into the defect area. (Figure 5B) In the group treated with doxycycline but without JFC scaffold, a lot of chondrocytes were seen adjacent to the bone tissue in the center part of the defect. These chondrocytes formed cluster mass with positive safranin O staining in the matrix of the cell cluster. (Figure 5C) The group with doxycycline combined with the JFC scaffold had a much smaller defect area than other groups. No positive stained safranin O in the defect area, but some fragments of degraded scaffold were seen embedded in the center part of the defect. (Figure 5D)

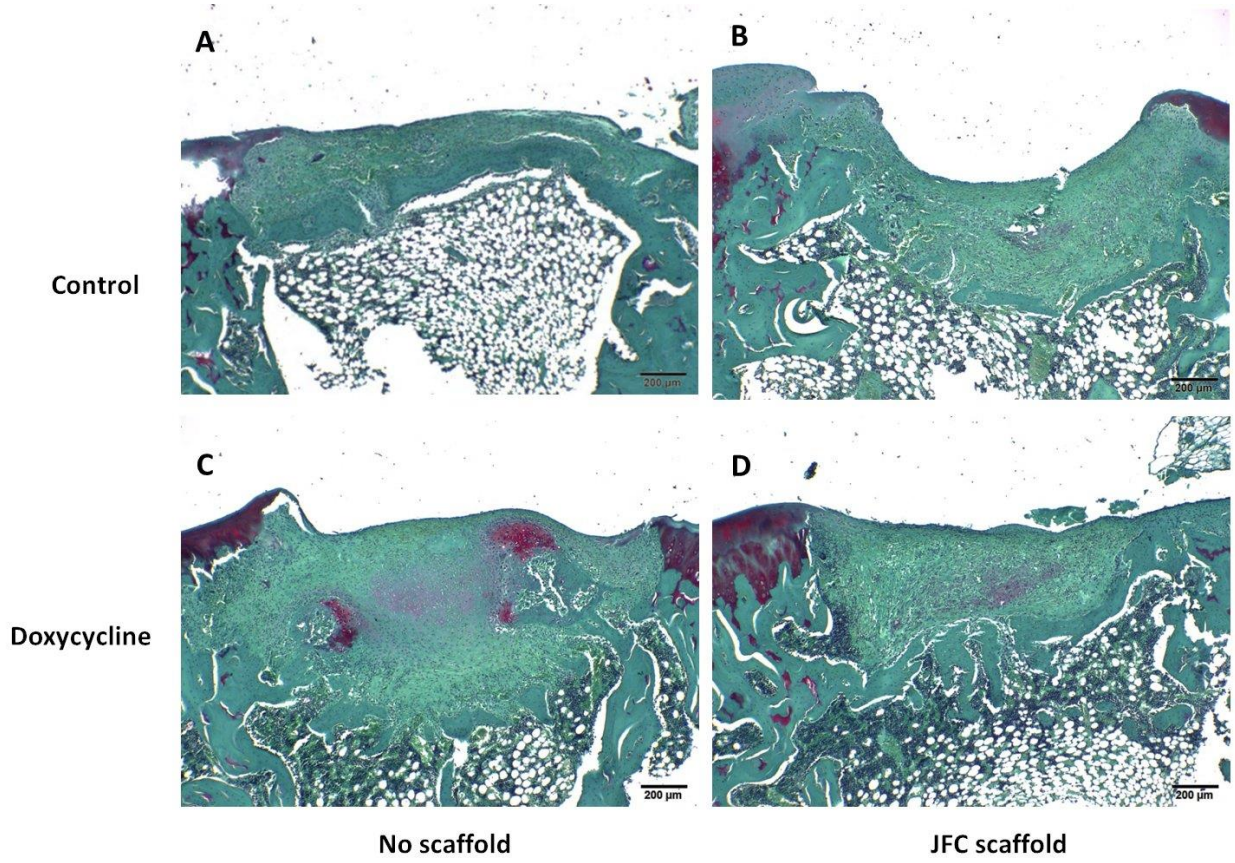

Figure 5. Saffranin O/fast green staining of cartilage and bone defect at 4 weeks post implantation. (A) No scaffold, No doxycycline. A layer of bone tissue was seen in the center of the defect connected between both sides. The area underneath of this bone tissue was a large hollow area filled by bone marrow cells. (B) JFC scaffold implanted, No doxycycline. Some fragment of scaffold (light purple color) was seen mixed with fibrous connective tissue in the center part of the defect. (C)Doxycycline treatment, No scaffold. The center part of the defect was seen containing red color cartilage like tissues. (D) Both of doxycycline treatment and JFC implantation. Some fragment of scaffold was seen in the center part of the defect.

At the 8-week time point, the control group still had fibrous tissue filling the defect. More bone tissue formed at the bottom of the defect. The size of the defect area was smaller than previously observed in the earlier time point. (Figure 6A) In the JFC scaffold only group, a piece of bone tissue was formed in the defect area. But fibrous tissue was also seen in the defect. The size of the defect was also smaller than the 4-week time point. (Figure 6B) A similar situation was seen in the doxycycline only group. The defect was filled by fibrous tissue and a piece of bone tissue growing out from the wall of the defect. (Figure 6C) However, the newly formed bone tissue in the group which received the combination of doxycycline and the JFC scaffold was more extensive than other groups and occupied the whole defect area with mature bone marrow and trabecular bone inside. (Figure 6D)

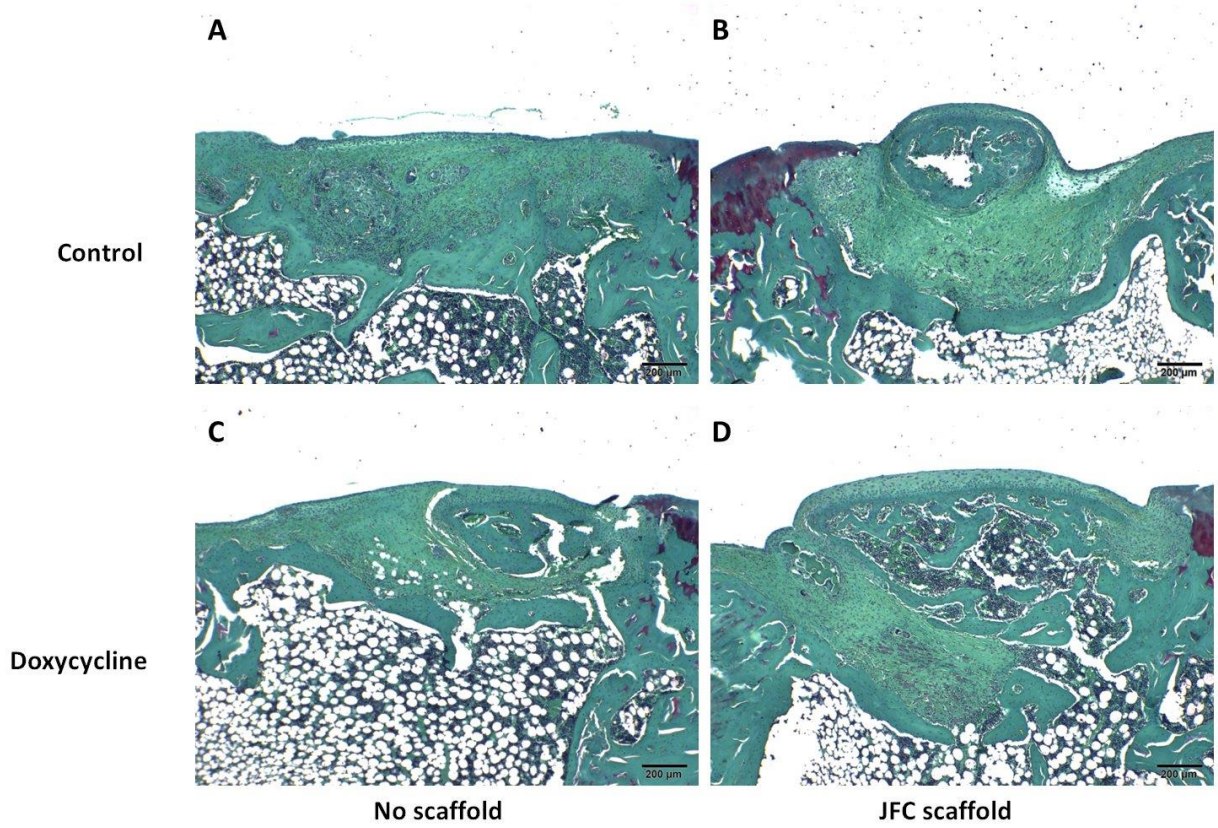

Figure 6. Safranin O/fast green staining of cartilage and bone defect at 8 weeks post implantation. (A) No scaffold, No doxycycline. The side and bottom area of the defect area was repaired by bone and bone marrow tissues. But the center and top area of the defect was filled with fibrous connective tissue. (B) JFC scaffold implanted, No doxycycline. A bony tissue was seen in the defect area separated from the bottom of the defect by fibrous connective tissues. (C)Doxycycline treatment, No scaffold. A bony tissue was seen connecting to the side of the defect and close to the surface but separated from the bottom bone tissue by fibrous connective tissue. (D) Both of doxycycline treatment and JFC implantation. A much large bone tissue was seen connected to the side of the defect but separated from the bottom bone tissue by fibrous connective tissue in the defect.

4. Discussion

4.1 The regulation of matrix metallopeptidases by doxycycline

In this present research, we studied the combination of doxycycline and JFC type II collagen scaffold for the repair of cartilage and bone defect in the knee joint. The effects of doxycycline on chondrocytes were tested with two different dosages in vitro. The human chondrocytes treated with high dose doxycycline (20μg/mL) showed significant decrease in the expressions of MMP1, MMP3, and MMP13. The inhibition of MMP1 and MMP13 was consistent with previously published reports.[2] Furthermore, our study demonstrated a decrease of MMP3 in chondrocytes observed both in low and high doses of doxycycline. Moreover, the decrease of MMP3 happened prior to inhibition of MMP1 and MMP13. MMP3 is one of the stromelysins.[12] It was reported that the administration of doxycycline decreased MMP3 in a rat tendon repair model at an early stage.[13] This present study is the first to report

the decrease of MMP3 expression in human chondrocytes after treatment with doxycycline. The protective effect of doxycycline on cartilage precedents have been reported.[14-17] However, most of the studies focused on the ability of doxycycline to inhibit collagenases, such as MMP1, MMP9, and MMP13.[2,18] The finding in the present study indicates that the effects of doxycycline on chondrocytes could be much broader. Fortier, et al. compared the expression of MMP3 from horse synoviocytes and horse cartilage tissues in a co-culture system with the treatment of doxycycline to antagonize the inflammatory stress induced by IL-1 and MMP13.[19] They found that the expression of MMP3 was decreased in synoviocytes after treatment with doxycycline at 4.3μM. However, the expression of MMP3 in the cartilage tissue fragments in the study was not influenced by doxycycline. Besides the difference in the species of animal model used, the co-culture system and the method employed to induce an in-vitro joint inflammatory environment might have diversely influenced the behavior of chondrocytes that resulted in the discrepancy between their report and this present study. This may indicate that accompanying change in the expression of MMP3 in chondrocytes after treatment with doxycycline might involve a more complex biological signaling system. In this present study, the expression of MMP9 was decreased with a low dosage of doxycycline at an early stage. However, the expression of MMP9 recovered at a later stage. The regulation of MMP9 expression is related to cell migration and angiogenesis in the tissue.[20] It is possible that the influence of doxycycline on the expression of MMP9 in human chondrocytes requires a specific adjustment within the cellular microenvironment.

4.2 The effects of promotion of progenitor and inhibition of maturation on chondrocytes by doxycycline

Besides its mediated influence on MMPs gene expressions, doxycycline is also involved in biological factors related to the growth of chondrocytes. In this study, after treatment with 20μg/mL doxycycline, the expression of type II collagen in human chondrocytes showed a decrease at all time points. TeKoppele, et al. had reported the expression of type II collagen in bovine chondrocytes to decline with increasing dosage of doxycycline in the medium.[21] Doxycycline was also reported to inhibit the synthesis of type X collagen.[22] It is unclear whether this inhibitory effect of doxycycline involves all types of collagen or specific subgroups. Our study also demonstrated an increase in the expression of Sox9; a change only observed at a later time point and not at an earlier time point. This is the first study to report such an effect of doxycycline on the gene expression of Sox9. Because Sox9 is an upstream transcription factor that is related to the growth of chondrocytes prior to the expression of type II collagen.[23,24] It is possible the increase of Sox9 was involved in the regulation of type II collagen expression. Furthermore, Sox9 is also a negative regulator of the expression of type X collagen.[25] Upregulated Sox9 might contribute to the inhibition of type X collagen expression by doxycycline. In this present study, both RunX2 and ALP decreased at a later time point with addition of doxycycline at 20μg/mL, although the expression of RunX2 initially increased at the earlier time point. Both these genes are considered as markers for mature or hypertrophic chondrocytes for tissue mineralization.[26-28] Doxycycline was reported to have caused the decrease in the activity of ALP in osteoprecursor cells.[29,30] This is the first study to confirm the expressions of both RunX2 and ALP in chondrocytes to have decreased after treatment with doxycycline.

4.3 Jellyfish type II collagen enhanced chondrogenesis of stem cells

To test the combinatory effect of collagen scaffold and doxycycline for the treatment of cartilage and bone defects in joints, JFC scaffolds were used in the in vivo portion of this study. From its component analyses, JFC is considered to be predominantly composed of type II collagen.[10] Since type II collagen is not only a major component of cartilage, but also plays a major contribution during endochondral bone formation. The application of JFC scaffolds might promote both cartilage and bone repairs. At first, this study tested its chondrogenesis inducing potential with human MSCs mixed with JFC particles. Both collagens type I and II, especially type II expression, increased with the mixture of JFC in the pellet culture. Furthermore, the expressions of MMP13 and DDR2 (hypertrophy chondrocyte markers [31,32]) decreased at the 2-week time point. These in vitro studies indicated that the presence of JFC improved the chondrogenic potential of MSCs and hindered their ossification in vitro. The chondrogenesis of MSCs was also confirmed by alcian blue staining. The enhanced chondrogenic potential of human MSCs cultured on JFC scaffolds has also been previously reported.[33,34] Furthermore, JFC has been reported to influence chondrocyte phenotype by preventing them from dedifferentiating.[10] When considering JFC for its tissue engineering application in cartilage repair, its characteristics, including the promotion of chondrogenesis of stem cells and inhibition of dedifferentiation or maturation of chondrocytes, makes it a suitable scaffold and biomaterial for maintaining biological performance of chondrocytes.

4.4 The combination of doxycycline and JFC for the repair of cartilage/bone defect in vivo

4.4.1 Doxycycline caused the accumulation of chondrocytes

The combination of doxycycline and JFC was evaluated by an in vivo experiment for the repair of a cartilage and bone defect created in knee joints. Because the critical defect size in rat knee joints is 1.4mm[35] , without additional processes, the defect created in this study (2mm) could not self-heal. The healing of bone tissue has three stages: early inflammation, repair, and remodeling.[36] The repair and remodeling stages happen almost simultaneously. When observing the control group at different time points, it showed the process of the bone reconstruction in the defect including both the repair and remodeling stages. The administration of doxycycline decelerated the process of bone formation, which caused the accumulation of chondrocytes in the defect observed at 4 weeks. However, more bone formation was observed in the defect than the control group at 8 weeks. This is the first study to demonstrate inhibition of bone formation at an early time but more bone formation at a later time after administration of doxycycline. A contradiction of the effect of bone repair after using doxycycline has been reported before. Both of Fawlkes, and Alkan groups reported no differences of bone formation with or without the administration of doxycycline in vivo.[37,38] However, some other groups reported the promotion of bone repair after using doxycycline.[4,5,39] It was considered that the mechanism of doxycycline on bone formation involves multiple factors, which interacted and influenced each other.[39] Since doxycycline was found to inhibit hypertrophy and maturation of chondrocytes in this study, it is possible that the endochondral bone formation was slowed down. The ossification of chondrocytes followed by early bone

formation could inhibit more bone marrow cells from infiltrating into the defect, suppressing the regeneration and remodeling of the tissue, which caused the accumulation of chondrocytes in the defects. These accumulated chondrocytes then formed a large piece of bone tissue in the defect later.

4.4.2 JFC degradation with more collagen deposition in the defect

The implantation of JFC also showed diminished bone formation after 4 weeks, but more bone formation in the defect than the control group at 8 weeks. Widdowson et al. reported the degradation of JFC almost totally finished within 4 weeks in vivo, with similar or even minor inflammatory reactions compared to bovine collagen scaffolds.[40] In this present study, nearly all of the JFC scaffold degraded and disappeared after 4 weeks. It was possible that the inflammation caused by JFC and simultaneous remodeling of the tissue restricted bone formation from occurring at an earlier time. Because the in vitro study showed the enhancement of both type I and II collagen expressions in MSCs and the potential inhibition of the maturation and hypertrophy of chondrocytes in the presence of JFC. The accumulation of both type I and II collagens in the extracellular matrix (ECM) created a platform to facilitate more bone deposition after the degradation of the scaffold.

4.4.3 The synergetic effect of bone formation promotion by doxycycline and JFC

Although the doxycycline or JFC groups had limited bone tissue formation than the control group at 4 weeks, they both demonstrated more bone tissue later. The indications of significant bone deposition in these results suggest that bone formation during the remodeling stage plays a major contribution to the repair of the defect. Furthermore, the enhanced bone formation seen in the defect associated with the experimental group that received a combination of doxycycline and JFC in comparison to other groups showed a potential synergistic effect of this combination in promoting bone tissue formation. It is possible that the implantation of the JFC scaffold provided more collagen accumulation in the ECM, while its biodegradation offered an opportunity for tissue remodeling to stimulate reconstruction. Meanwhile, the administration of doxycycline inhibited the ossification of chondrocytes, which allowed their proliferation and accumulation in the defect. The ossification of the tissue with more chondrocytes and more collagens in ECM subsequently augmented bone formation in the defect.

4.5. Neither doxycycline nor JFC enhanced the cartilage repair in the defects

In contrast to the enhanced bone tissue repair observed within the defect, neither the administration of doxycycline, nor the implantation of JFC improved the cartilage tissue repair. The contribution of more collagen and chondrocytes in the defect turned into bone formation rather than the cartilage tissue formation. This result may suggest that the direct migration of bone marrow cells at the local site of articular defects is not sufficient to initiate the repair of cartilage tissue. The contribution of biodegradable scaffolds or regulation of the metabolism of the tissue may require alternative ways to stimulate and mobilize cells from within the different cartilage layers, but not from bone marrow, to mediate the repair of cartilage. It was considered that the formation of the cartilage layer relied on a bidirectional migration of chondrocytes within the cartilage between the superficial layer and the deep zone.[41] For the restoration of the cartilage tissue, it has to rebuild the zonal structures within the interaction between the cartilage and bone, which includes the hyaline cartilage, tidemark, calcified cartilage layer and subchondral

bone.[41-43] And these structures are developed from the interaction between the chondrocytes in the cartilage, and osteocytes and bone marrow cells in the subchondral bone tissue, and confer specialized functions in maintaining the articular cartilage. As demonstrated in this present study, without these layered structures within the cartilage/bone interaction, the mediation of articular and subchondral bone defect repair applicable during the process of the local tissue restoration will only contribute to the bone tissue repair.

5. Conclusion

This study tested using doxycycline and JFC scaffold for the repair of a cartilage and bone defect in a rat model. Doxycycline was found to inhibit the ossification of chondrocytes. JFC had the capacity of promoting the accumulation of collagens in the extracellular matrix. The applications of doxycycline or JFC alone enhance the bone formation in the defect. The combination of doxycycline and JFC had a synergistic effect of bone formation. However, the cartilage repair was not promoted even with improved accumulation of collagens and chondrocytes within the defect site. It is possible that the reconstruction of the zonal structures maintaining the interaction between the cartilage and bone is necessary for the repair of the cartilage layer itself.

References

1. Lee HH, O'Malley MJ, Friel NA, Chu CR. Effects of doxycycline on mesenchymal stem cell chondrogenesis and cartilage repair. *Osteoarthr Cartil*. 2013;21(2):385–393. doi:10.1016/j.joca.2012.11.010

2. Shlopov BV, Stuart JM, Gumanovskaya ML, Hasty KA. Regulation of cartilage collagenase by doxycycline. *J Rheumatol*. 2001;28(4):835–842.

3. Bowyer J, Heapy CG, Flannelly JK, Waterton JC, Maciewicz RA. Evaluation of a magnetic resonance biomarker of osteoarthritis disease progression: doxycycline slows tibial cartilage loss in the Dunkin Hartley guinea pig. *Int J Exp Pathol*. 2009;90(2):174–181. doi:10.1111/j.1365-2613.2008.00634.x

4. Gomes KdN, Alves APNN, Dutra PGP, Viana GSdB. Doxycycline induces bone repair and changes in Wnt signalling. *Int J Oral Sci*. 2017;9(3):158–166. doi:10.1038/ijos.2017.28

5. Muthukuru M, Sun J. Doxycycline counteracts bone morphogenic protein 2-induced osteogenic mediators. *J Periodontol*. 2013;84(5):656–665. doi:10.1902/jop.2012.120338

6. Aydin O, Korkusuz F, Korkusuz P, et al. In vitro and in vivo evaluation of doxycycline-chondroitin sulfate/PCLmicrospheres for intraarticular treatment of osteoarthritis. *J Biomed Mater Res Part B Appl Biomater*. 2015;103(6):1238–1248. doi:10.1002/jbm.b.33303

7. Bermueller C, Schwarz S, Elsaesser AF, et al. Marine collagen scaffolds for nasal cartilage repair: prevention of nasal septal perforations in a new orthotopic rat model using tissue engineering techniques. *Tissue Eng Part A*. 2013;19(19-20):2201–2214. doi:10.1089/ten.TEA.2012.0650

8. Hadzik J, Kubasiewicz-Ross P, Kunert-Keil C, et al. A silver carp skin derived collagen in bone defect treatment-A histological study in a rat model. *Ann Anat*. 2016;208:123–128. doi:10.1016/j.aanat.2016.07.009

9. Addad S, Exposito J-Y, Faye C, Ricard-Blum S, Lethias C. Isolation, characterization and biological evaluation of jellyfish collagen for use in biomedical applications. *Mar Drugs*. 2011;9(6):967–983. doi:10.3390/md9060967

10. Sewing J, Klinger M, Notbohm H. Jellyfish collagen matrices conserve the chondrogenic phenotype in two- and three-dimensional collagen matrices. *J Tissue Eng Regen Med*. 2017;11(3):916–925. doi:10.1002/term.1993

11. Shah SS, Liang H, Pandit S, et al. Optimization of Degradation Profile for New Scaffold in Cartilage Repair. *Cartilage*. 2018;9(4):438–449. doi:10.1177/1947603517700954

12. Burrage PS, Mix KS, Brinckerhoff CE. Matrix metalloproteinases: role in arthritis. *Front Biosci*. 2006;11:529–543. doi:10.2741/1817

13. Nguyen QT, Norelli JB, Graver A, et al. Therapeutic Effects of Doxycycline on the Quality of Repaired and Unrepaired Achilles Tendons. *Am J Sports Med*. 2017;45(12):2872–2881. doi:10.1177/0363546517716637

14. Zhang X, Deng X-H, Song Z, et al. Matrix Metalloproteinase Inhibition With Doxycycline Affects the Progression of Posttraumatic Osteoarthritis After Anterior Cruciate Ligament Rupture: Evaluation in a New Nonsurgical Murine ACL Rupture

Model. *Am J Sports Med*. 2020;48(1):143–152. doi:10.1177/0363546519887158

15. Yu LP, Smith GN, Brandt KD, Myers SL, O'Connor BL, Brandt DA. Reduction of the severity of canine osteoarthritis by prophylactic treatment with oral doxycycline. *Arthritis Rheum*. 1992;35(10):1150–1159. doi:10.1002/art.1780351007

16. Rabillard M, Danger R, Doran IP, Niebauer GW, Brouard S, Gauthier O. Matrix metalloproteinase activity in stifle synovial fluid of cranial cruciate ligament deficient dogs and effect of postoperative doxycycline treatment. *Vet J*. 2012;193(1):271–273. doi:10.1016/j.tvjl.2011.10.028

17. Brandt KD, Mazzuca SA, Katz BP, et al. Effects of doxycycline on progression of osteoarthritis: results of a randomized, placebo-controlled, double-blind trial. *Arthritis Rheum*. 2005;52(7):2015–2025. doi:10.1002/art.21122

18. Ghasemi S, Sardari K, Mirshokraei P, Hassanpour H. In vitro study of matrix metalloproteinases 1, 2, 9, 13 and serum amyloid A mRNAs expression in equine fibroblast-like synoviocytes treated with doxycycline. *Can J Vet Res*. 2018;82(2):82–88.

19. Fortier LA, Motta T, Greenwald RA, Divers TJ, Mayr KG. Synoviocytes are more sensitive than cartilage to the effects of minocycline and doxycycline on IL-1alpha and MMP-13-induced catabolic gene responses. *J Orthop Res*. 2010;28(4):522–528. doi:10.1002/jor.21006

20. Jabłońska-Trypuć A, Matejczyk M, Rosochacki S. Matrix metalloproteinases (MMPs), the main extracellular matrix (ECM) enzymes in collagen degradation, as a target for anticancer drugs. *J Enzyme Inhib Med Chem*. 2016;31(sup1):177–183. doi:10.3109/14756366.2016.1161620

21. TeKoppele JM, Beekman B, Verzijl N, Koopman JL, DeGroot J, Bank RA. Doxycycline inhibits collagen synthesis by differentiated articular chondrocytes. *Adv Dent Res*. 1998;12(2):63–67. doi:10.1177/08959374980120012201

22. Davies SR, Cole AA, Schmid TM. Doxycycline inhibits type X collagen synthesis in avian hypertrophic chondrocyte cultures. *J Biol Chem*. 1996;271(42):25966–25970. doi:10.1074/jbc.271.42.25966

23. Ng LJ, Wheatley S, Muscat GE, et al. SOX9 binds DNA, activates transcription, and coexpresses with type II collagen during chondrogenesis in the mouse. *Dev Biol*. 1997;183(1):108–121. doi:10.1006/dbio.1996.8487

24. Bell DM, Leung KK, Wheatley SC, et al. SOX9 directly regulates the type-II collagen gene. *Nat Genet*. 1997;16(2):174–178. doi:10.1038/ng0697-174

25. Leung VYL, Gao B, Leung KKH, et al. SOX9 governs differentiation stage-specific gene expression in growth plate chondrocytes via direct concomitant transactivation and repression. *PLoS Genet*. 2011;7(11):e1002356. doi:10.1371/journal.pgen.1002356

26. Chen H, Ghori-Javed FY, Rashid H, et al. Runx2 regulates endochondral ossification through control of chondrocyte proliferation and differentiation. *J Bone Miner Res*. 2014;29(12):2653–2665. doi:10.1002/jbmr.2287

27. Orimo H. The mechanism of mineralization and the role of alkaline phosphatase in health and disease. *J Nippon Med Sch*. 2010;77(1):4–12. doi:10.1272/jnms.77.4

28. Komori T. Runx2, an inducer of osteoblast and chondrocyte differentiation. *Histochem Cell Biol*. 2018;149(4):313–323. doi:10.1007/s00418-018-1640-6

29. Park J-B. Effects of doxycycline, minocycline, and tetracycline on cell proliferation, differentiation, and protein expression in osteoprecursor cells. *J Craniofac Surg*. 2011;22(5):1839–1842. doi:10.1097/SCS.0b013e31822e8216

30. Gomes PS, Fernandes MH. Effect of therapeutic levels of doxycycline and minocycline in the proliferation and differentiation of human bone marrow osteoblastic cells. *Arch Oral Biol*. 2007;52(3):251–259. doi:10.1016/j.archoralbio.2006.10.005

31. Tchetina EV, Kobayashi M, Yasuda T, Meijers T, Pidoux I, Poole AR. Chondrocyte hypertrophy can be induced by a cryptic sequence of type II collagen and is accompanied by the induction of MMP-13 and collagenase activity: implications for development and arthritis. *Matrix Biol*. 2007;26(4):247–258. doi:10.1016/j.matbio.2007.01.006

32. Zhang S, Zhong Y, Li R, et al. Experimental chondrocyte hypertrophy is promoted by the activation of discoidin domain receptor 2. *Mol Med Rep*. 2014;10(3):1543–1548. doi:10.3892/mmr.2014.2340

33. Pustlauk W, Paul B, Brueggemeier S, Gelinsky M, Bernhardt A. Modulation of chondrogenic differentiation of human mesenchymal stem cells in jellyfish collagen scaffolds by cell density and culture medium. *J Tissue Eng Regen Med*. 2017;11(6):1710–1722. doi:10.1002/term.2065

34. Hoyer B, Bernhardt A, Lode A, et al. Jellyfish collagen scaffolds for cartilage tissue engineering. *Acta Biomater*. 2014;10(2):883–892. doi:10.1016/j.actbio.2013.10.022

35. Katagiri H, Mendes LF, Luyten FP. Definition of a Critical Size Osteochondral Knee Defect and its Negative Effect on the Surrounding Articular Cartilage in the Rat. *Osteoarthr Cartil*. 2017;25(9):1531–1540. doi:10.1016/j.joca.2017.05.006

36. Kalfas IH. Principles of bone healing. *Neurosurg Focus*. 2001;10(4):E1. doi:10.3171/foc.2001.10.4.2

37. Fowlkes JL, Nyman JS, Bunn RC, et al. Effects of long-term doxycycline on bone quality and strength in diabetic male DBA/2J mice. *Bone Rep*. 2015;1:16–19. doi:10.1016/j.bonr.2014.10.001

38. Alkan A, Erdem E, Günhan O, Karasu C. Histomorphometric evaluation of the effect of doxycycline on the healing of bone defects in experimental diabetes mellitus: a pilot study. *J Oral Maxillofac Surg*. 2002;60(8):898–904. doi:10.1053/joms.2002.33859

39. Shahabooei M, Razavi SM, Minaiyan M, et al. A histomorphometric study of the effect of doxycycline and erythromycin on bone formation in dental alveolar socket of rat. *Adv Biomed Res*. 2015;4:71. doi:10.4103/2277-9175.153895

40. Widdowson JP, Picton AJ, Vince V, Wright CJ, Mearns-Spragg A. In vivo comparison of jellyfish and bovine collagen sponges as prototype medical devices. *J Biomed Mater Res Part B Appl*

Biomater. 2018;106(4):1524–1533. doi:10.1002/jbm.b.33959

41. Simkin PA. Consider the tidemark. *J Rheumatol*. 2012;39(5):890–892. doi:10.3899/jrheum.110942

42. Lyons TJ, McClure SF, Stoddart RW, McClure J. The normal human chondro-osseous junctional region: evidence for contact of uncalcified cartilage with subchondral bone and marrow spaces. *BMC Musculoskelet Disord*. 2006;7:52. doi:10.1186/1471-2474-7-52

43. Lyons TJ, Stoddart RW, McClure SF, McClure J. The tidemark of the chondro-osseous junction of the normal human knee joint. *J Mol Histol*. 2005;36(3):207–215. doi:10.1007/s10735-005-3283-x

