공학석사 학위논문

Inflammation-regulated hyaluronic acid hydrogel for osteoarthritis cartilage repair

골 관절염 연골 치료를 위한 염증조절형

히알루론산 하이드로겔

2019년 2월

서울대학교 대학원

화학생물공학부 화학생물공학과

Yin Ji Jin

Inflammation-regulated hyaluronic acid hydrogel for osteoarthritis cartilage repair

골 관절염 연골 치료를 위한 염증조절형

히알루론산 하이드로겔

지도 교수 황 석 연

이 논문을 공학석사 학위논문으로 제출함

2019년 1월

서울대학교 대학원

화학생물공학부 화학생물공학과

Yin Ji Jin

Yin Ji Jin의 공학석사 학위논문을 인준함

2019년 2월

위 원 장 김병기 교수님 (인)

부위원장 황석연 교수님 (인)

위 원 김병수 교수님 (인)

Abstract

Inflammation-regulated hyaluronic acid hydrogel for osteoarthritis cartilage repair

Yin Ji Jin

School of Chemical and Biological Engineering

The Graduate School

Seoul National University

Osteoarthritis (OA) is the most common musculoskeletal disorder that has limited regenerative capacity once damaged. Once OA is induced in the joint, the subsequent immune reaction leads to chronic inflammation that results in the progression of OA and deconstruction of the cartilage. In this study, an injectable hydrogel with Epigallocatechin-3-gallate (EGCG) is introduced to control inflammation and enhance cartilage regeneration. EGCG has intrinsic properties that can modulate inflammation and scavenge radical species. Hyaluronic acid (HA) is a major component of the cartilage ECM, and therefore, commonly used for cartilage tissue engineering. In this study, EGCG was combined with tyramine-conjugated HA and gelatin to create a composite hydrogel with an optimal concentration of 50 μM and 5% *w/v* HA. The composite hydrogel provided

protection to chondrocytes against pro-inflammatory factor, IL-1β. Additionally, the composite hydrogel led to chondrogenic regeneration *in vitro*. Histological analysis *in vivo* showed that EGCG-HA/Gelatin hybrid hydrogel minimized cartilage loss in surgically induced OA model. This study demonstrates that inflammation-modulating HA-based hydrogel may provide a therapeutic option for OA treatment.

Keyword : osteoarthritis, tissue engineering, anti-inflammation, EGCG, hyaluronic acid, hydrogel
Student Number : 2016-23954

Table of Contents

Lists of Figures and Tables

CHAPTER 1: SCIENTIFIC BACKGROUND

1.1 Pathogenesis of osteoarthritis

Osteoarthritis(OA) is one of the most common musculoskeletal diseases that afflict 240 million people worldwide [1]. Generally, OA affects most adults aged 65 or older, also occurs in people who exercise a lot, such as athletes. In normal joints, cartilage, covers the end of each bone, acts as a cushion between the bones and provides a smooth, gliding surface for joint motion, which facilitated by a boundary layer of lubricant consist of hyaluronic acid and lubricin [2]. In OA, cartilage breaks down and becomes thinner because of that interacting trauma, mechanical forces, inflammation, biochemical reactions, and metabolic derangements [3]. In severe cases, cartilage completely wears down and bones rub against one another causing severe pain and inflammation.

Traditionally, OA has been regarded only as an age-related degenerative disease only, for which no medical treatment besides joint replacement surgery. Recently, it has been confirmed that OA is reclassified as an inflammatory, systemic disease with abnormal metabolic overtones through many systematic analyses of sera, synovial tissue, synovial fluid, articular cartilage, and subchondral bone.

In OA joint, there is cartilage degradation induced by loss of proteoglycans and breakdown of collagen network of the extracellular matrix (ECM) [4, 5].

This reduction in ECM is mainly due to pro-inflammatory cytokines, such as interleukin 1-beta (IL-1b) and tumor necrosis factor (TNF-a) that play a pivotal role in OA pathogenesis [6]. Such upstream pro-inflammatory cytokines activate the NF-κB signaling pathway, causing systemic inflammation on all the tissues around the joints such as cartilage, synovial membrane, subchondral bone and ligaments [7, 8]. Chondrocytes, resident cells of the cartilage, experience great stress in inflammatory environment and undergo various phenotypical changes. Compared to healthy cells, OA chondrocytes produce elevated levels of pro-inflammatory cytokines TNF-α, IL-1b, and IL-6, and ECM degradative enzymes, including matrix metalloproteinases (MMPs), and aggrecanases [9, 10], while producing low levels of ECM molecules. Finally, the NF-κB molecule activates nitric oxide (NO), cyclooxygenase 2 (COX-2), nitric oxide synthase (NOS), and prostaglandin E2 (PGE2), which promote catabolism, resulting in cartilage inflammation and OA chondrocytes apoptosis [11].

1.2 Current strategies for OA treatment

1.2.1 Pharmacological treatments

Drugs for OA treatment focus on reducing pain and inflammation. Corticosteroid drugs (CSDs) and non-steroid anti-inflammatory drugs (NSAIDs) are the most commonly used medications for OA. NSAIDs are medications that are helpful in reducing pain, decreasing fever and

inflammation and preventing blood clots. Most NSAIDs have side effects when used for a prolonged period of time, which increases the risk of gastrointestinal bleeding ulcers, heart attack and kidney disease [12]. NSAIDs exhibit anti-inflammatory and antipyretic effects by blocking prostanoid production from arachidonic acid via cyclooxygenase enzyme inhibition [13]. CSDs are powerful anti-inflammatory medicines that can reduce pain and inflammation in OA [14]. Since CSDs are hormones, they have a wide range of side effects. The long-term administration of CSDs increases the risk of hypertension, diabetes, and osteoporosis [15].

Oral administration of CSDs or NSAIDs is the most commonly prescribed anti-inflammatory treatment for OA, to relieve pain and inflammation. Oral administration of these therapeutic agents is transported to the intra-articular site of action via systemic circulation. It takes 5-6 hours for the orally administered drugs to move to the joints and reach the highest concentration [16]. Concentrations of drugs in the synovial fluid are considered to be important determinants of the clinical efficacy of the drugs [17]. Direct intra-articular injection of the drugs can overcome the problems of low concentration drug delivery via oral administration, and only a minimum amount of the drug is required to exert the desired pharmacological activity. However, there is the problem of rapid clearance of the dissolved drugs from the synovial fluid. In general, the duration of the soluble drug in the synovial fluid was only for a few hours after intra-articular injection [18].

Maintaining the concentration of the therapeutic drug over a prolonged period of time could be achieved by repeated intra-articular administration or more ideally, by immobilizing the active agent in the form of injectable depot formulations in which the drug is released in a controlled manner.

1.2.2 Viscosupplementation treatments

Viscosupplementation (VS) intra-articular injections of hyaluronic acid (HA) and lubricin (proteoglycan 4, PRG 4), are widely used for symptomatic knee OA. Hyaluronic acid is the most commonly used biomaterial for VS. HA is the major component of synovial fluid and well-recognized as a glycosaminoglycan (GAG), a major component of the extracellular matrix (ECM). Intra-articular injection of HA provides relief from symptoms through a variety of different mechanisms such as enhancing the synthesis of extracellular matrix proteins, altering inflammatory mediators in order to shift away from degradation, reducing friction of the joints, and maintaining cartilage thickness, and surface smoothness [19]. It has been reported that HA had a greater effect on pain relief than a placebo [20, 21]. OA patients with intra-articular injections of HA for every 6 months showed a beneficial effect on knee cartilage preservation compared to those without HA injection [22].

In a physiological environment, HA is subjected to various degradation processes due to hydrolysis and also enzymatic hydrolysis by naturally

occurring hyaluronidase. The turnover of HA in joints is surprisingly rapid [23]. Therefore, it is required to establish strategies that improve the mechanical properties and control the degradation rate of HA.

1.2.3 Cell-based treatments

Compared to pharmacological and VS treatments, which are symptomatic regulatory therapies, cell-based treatments are more focused on regenerative procedures. To replace or repair damaged tissues, stem, progenitor or primary cells are utilized as a regenerative strategy [24]. The effect of cell-based therapy can be realized through two mechanisms.

One of the mechanisms of cell therapy is based on the replacement of injured cells or tissue by engraftment into the damaged tissue. The basic cell-based OA treatment is autologous chondrocyte implantation (ACI). Autologous chondrocytes isolated from the cartilage that are harvested from a non-weight bearing area, are implanted to the affected area [25]. Carticel® (Genzyme), which is FDA approved, provides autologous cultured chondrocytes to repair the cartilage [26]. Despite encouraging clinical results, there are still limitations to the use of ACI due to the complexity and cost of two surgical procedures, and the de-differentiation and consequent loss of capacity associated with *in vitro* expansion of isolated chondrocytes.

Interest in mesenchymal stem cells (MSCs) as a treatment strategy for OA is increasing considerably. MSCs are multipotent adult stem cells with the

capacity to differentiate to osteoblasts, chondrocytes, and adipocytes [27]. MSCs also secrete a range of molecules, such as trophic factors, cytokines, and neuroregulatory peptides, which play a role in tissue repair and regulate inflammatory and immune responses. CARTISTEM® (Medipost) has been approved by the Ministry of Food & Drug Safety in South Korea, which provides allogenic umbilical cord, blood-derived mesenchymal stem cells (UCB-MSCs) with HA to repair cartilage [28]. Recently, JointStem® (RNL), an OA treatment which uses adipose tissue-derived mesenchymal stem cells (AD-MSCs) entered phase I/II clinical trials designed to treat patients with degenerative arthritis [29].

Another mechanism is the stimulation of endogenous, tissue self-healing processes through trophic effects mediated by cytokine and growth factor secretions. Recent studies have found that stem cells can also treat OA through paracrine mechanisms [30]. Stem cells secrete cytokines which can regulate inflammatory conditions, or growth factors that can increase regenerative properties [31]. MSCs are often considered as 'environmentally-responsive' cells and they are known to secrete bioactive factors and signals in response to local microenvironmental cues [32].

1.2.4 Gene therapies

The Ministry of Food and Drug Safety of South Korea has approved the first gene therapy, TissueGene Invossa (TissueGene-C) for OA treatment.

Invossa consists of a fixed-ratio mixture of non-transduced allogeneic human chondrocytes and allogeneic human chondrocytes expressing transforming growth factor beta 1 (TGF-β1). TGF-β1-stimulated chondrocytes play a critical role in the formation and maintenance of articular cartilage. Invossa has the ability to stimulate the differentiation of M2 macrophages and interrupt the innate immune response. It has been confirmed that Invossa reduced the symptoms of OA patients by 84% and 88% in a Korea-based phase III trial and a US-based phase II trial, respectively and also the reduced the OA symptoms lasting for up to two years. [33].

1.3 Inflammatory-modulating biomaterials for OA

OA is no longer considered a “wear and tear” condition but is rather driven by chronic inflammation which might play a role in ECM degradation. It is expected that the combination of current anti-inflammatory therapies and biomaterials will effectively modulate the inflammation and regeneration of OA cartilage. Hyaluronic acid hydrogel has been used as the vehicle for delivering doxycycline, one of the NSAIDs, to the joint. The release rate of doxycycline in HA hydrogel was determined by the molecular weight (Mw) of HA. Doxycycline, with a larger molecular weight, was released slowly than the smaller molecule HA, because of rapid gelation. This showed that the release rate could be controlled by the

molecular weight of polymers [34, 35]. Audrey et al. demonstrated an acetyl-capped PCLA-PEG-PCLA composite hydrogel which was loaded with celecoxib, a COX-2 inhibitor NSAID. In order to slow down the hydrolysis and increase the degradation time, PCLA was used instead of PLGA, and the degradation rate can be adjusted by calculating the ratio of caproyl units (CL) to lactoyl units (LA). This system was well tolerated after intra-articular administration and showed local drug administration which generates high local doses without the risk of side effects associated with oral administration [36]. Laurent et al. demonstrated sustained delivery of NSAIDs without initial burst release. They produced hydrophilic degradable microspheres where ibuprofen is conjugated via an ester linkage to a polymethacrylate backbone. Microspheres (MS) (40–100 μm) showed a low yield of ibuprofen release in saline buffer (≈ 2% after 3 months). This study represents that incorporation of ibuprofen prodrug within a PEG-hydrogel MS suppressed as expected, the initial burst release and allowed long-term drug delivery [37]. Dexamethasone, a classic corticosteroid, is commonly administered orally to treat OA. A cross-linked hyaluronic acid hydrogel was constructed and loaded with dexamethasone. The loading process was quite simple; dexamethasone solvent was mixed with HA solvent before gelation. This hydrogel allowed the slow release of dexamethasone into the joint cavity, where it manifested its anti-inflammatory role [38].

CHAPTER 2: INFLMMATION-REGULATED HYALURONIC ACID HYDROGEL FOR OSTEOARTHRITIS CARTILAGE REPAIR

2.1 Introduction

Osteoarthritis (OA) is known as the most common chronic disease of the cartilage. It usually happens with age and is commonly induced by extraordinary loading pressure or traumatic injury of the joint. When cartilage wears and swells with the progression of OA, it causes pain and inflammation, leading to extracellular matrix (ECM) loss, such as collagen and glycosaminoglycan (GAG). The imbalance between anabolic and catabolic processes destroys cartilage's normal ECM maintenance [39, 40]. The main catabolic cytokine involved in the OA pathogenesis is Interleukin-1 beta (IL-1β). OA patients have elevated levels of IL-1β in the synovium and cartilage [41]. IL-1β significantly affect the metabolism of the chondrocytes and its ECM. A previous study by Stove *et al*., has demonstrated that IL-1β blocks the synthesis of ECM components such as type II collagen (Col II) and aggrecan (ACAN) [42]. Thus, medications that target inflammation of joint cartilage deserve attention in therapeutic strategies that delay OA progression.

Nowadays, many researchers have increased attention to polyphenol

that is a pharmaceutical-grade nutrient. It has been reported that polyphenols have many therapeutic effects such as anti-cancer, anti-oxidant, and anti-inflammation for many diseases [43-46]. Epigallocatechin-3-gallate (EGCG) is one of the polyphenols, most abundantly found in green tea. EGCG has been shown to inhibit inflammation caused by various cell types, such as immune cells, vascular endothelial cells, fibroblasts, and chondrocytes [44, 47, 48]. Over the last decade, several studies have demonstrated the inflammatory regulation and chondroprotective effects of EGCG on monolayer cultured chondrocytes [49-51]. EGCG has been shown to inhibit metalloproteinases (*MMP13*) cyclooxygenase-2 (*COX-2*), and nuclear factor-kappa B (*NF-kB*) in IL-1-stimulated chondrocytes [52-54].

Current OA treatments are limited to alleviate the symptoms of OA by taking some analgesics and nonsteroidal anti-inflammatory drugs (NSAIDs), or intra-articular injection of steroid drugs[55]. These treatments, when carried out long-term, have detrimental side effects on gastrointestinal and cardiovascular systems. Therefore, OA therapy still needs development. The on-site delivery of drugs via controlled releasing systems is a new breakthrough. For the delivery system, hyaluronic acid (HA)-based hydrogel has been established as a useful vehicle for drug delivery [56]. HA is a substance that occurs naturally in the joints and is known as a major component of GAG. HA is widely used in many tissue engineering approaches because of its biocompatibility, biodegradability and low

immunogenicity. Since the elimination of injected HA occurs rapidly, HA is usually crosslinked to last longer [57, 58]. So, HA-based hydrogels are widely studied for OA treatment. However, HA-based hydrogels have limitation cell adhesion [59]. Gelatin provide good cell adherent environment [60]. In this study, we fabricated hybrid hydrogels that consist of HA and gelatin to mimic the cartilage ECM. For HA and gelatin hydrogel formation, HA was conjugated with tyramine (HA_t) to crosslink via tyrosine-induced crosslinking of tyramine residues [61]. Recently, we have reported enzyme-based hydrogel crosslinking systems with tyrosinase from *Streptomyces avermitillis* (SA_Ty) [62]. SA_Ty-mediated HA_t hydrogel has advantages., including rapid crosslinking, non-cytotoxicity, and injectability.

In this study, we investigated the EGCG-loaded HA/Gelatin (HTG-E) hybrid hydrogel for OA therapy. We first studied effects of EGCG and HA/Gelatin (HTG) hybrid hydrogel on chondrocytes, independently. The evaluation of the anti-inflammatory effect of EGCG was performed by OA-like chondrocyte laden hydrogel. The chondrogenic potential of HTG hydrogel was investigated by cartilage formation for 3 weeks *in vitro*. Finally, we combined the composite hydrogel with EGCG to provide a suitable 3-dimensional (3D) environment for the growth of inflammation-induced chondrocyte *in vitro*. The *in vivo* evaluation of EGCG-loaded composite hydrogel demonstrated a synergetic anti-inflammatory and

chondroprotective effects on OA progression. We have established a novel method for OA therapy with inflammation-modulating HA and gelatin-based hybrid hydrogels.

2.2 Experimental section

2.2.1 Porcine articular chondrocyte isolation

Articular cartilage was dissected from femoral condyles which were purchased from a local slaughterhouse. Cartilage tissue was minced into 1mm^3 pieces and digested with 0.2% (*w/v*) type II collagenase for 18h on an orbital shaker at 37℃. Subsequently, the digested cell suspension was strained by 100μm cell strainers and centrifuged. The cell pellet was washed with PBS 2-3 times and culture in high glucose Dulbecco's modified Eagles' medium, supplemented with 10% FBS, 1% penicillin/streptomycin, 10 mM HEPES, 0.1 mM NEAA, 0.4 mM proline, 50 μg mL^{-1} ascorbic acid.

2.2.2 Hydrogel preparation

HA_tyramine (HA_t) synthesis was performed by EDC/NHS coupling protocol which was demonstrated by previous work [61]. Briefly, Sodium hyaluronate (Lifecore Biomedical) was dissolved into 2-(N-morpholino) ethanesulfonic acid (MES) buffered saline (Thermo Fisher Scientific) to make 1% (w/v) of HA solution. While stirring, Nhydroxysulfosuccinimide

(sulfo-NHS; Sigma Aldrich) and N-(3-dimethylaminopropyl)-N-ethylcarbodiimide (EDC; Sigma Aldrich) were added to the HA solution. Tyramine hydrochloride (Sigma Aldrich) was added with the same molar of HA. The reaction was performed overnight, and dialysis against distilled water for 3 days. Synthesized HA_t was lyophilized for further use.

Dissolve HA_t powder in PBS to make 4% or 10% (*w/v*) HA_t solutions and dissolve gelatin in PBS to make 10% (*w/v*) gelatin solutions. Then solutions were sterilized by the 0.25 μm syringe filters and mixed in 1:1 ration to make 2% HTG, and 5% HTG hydrogel precursors. 0% HTG hydrogel precursor was composed of 5% (*w/v*) gelatin solution only (without HA_t solution). 6 μM of SA_Ty was added into the precursors and induced enzymatic crosslinking [62].

2.2.3 Rheological test

To measure the rheological properties, the hydrogels were fabricated in 8mm diameter and 2 mm thickness cylindrical shape. The test was carried out in Demo lab (Anton Paar Korea) using rheometer (MCR 302, Measuring cell: P-PTD & H-PTD 200, Measuring System: PP 25, Anton-Paar, Austria). The storage modulus G' of amplitude sweep was measured with increasing shear strain (%) from 0.1 to 10 HZ. Next, the frequency sweep was performed with increasing frequency from 0.1 to 10 HZ at a constant strain of 1% to investigate the modulus change.

2.2.4 Swelling ratio

For measuring the swelling ratio (Q), the hydrogels (*n*=3) were immersed in PBS for 24 hours. The swollen weight (W_{wet}) of hydrogels was measured, and then lyophilized, the dry weight (W_{dry}) was measured, too. Swelling ratio was calculated by the following equation.

$$Q = \frac{Wwet}{Wdry} \quad (1)$$

2.2.5 Enzymatic degradation test

Hydrogels were prepared at same volume (50 μL/construct; *n*=3/time point). 10 unit of hyaluronidase and collagenase were used independently at this test and was changed every day. Each time-point samples were collected and lyophilized, and the dry weight was measured. The weight loss of dry weight of hydrogel was calculated.

2.2.6 Cell encapsulation

Articular chondrocytes (Passage 1-2) were prepared for cell 3D encapsulation. Cells were mixed with hydrogel precursors and added 6 μM of SA_Ty for enzyme crosslinking in PDMS mold. Final cell concentration in hydrogel was $2x10^7$ cells mL^{-1}. After gelation, cell-laden hydrogels were transferred to 24 well-plate and supplied with high glucose Dulbecco's modified Eagles' medium (DMEM, Corning), supplemented with 1%

penicillin/streptomycin (Gibco), 1% ITS (BD Biosciences), 100nM Dexamethasone (Sigma Aldrich), 50 mg mL^{-1} ascorbic acid-2-phosphate (Sigma Aldrich), and 10 ng mL^{-1} TGF-β3 (Life technologies). The media was changed every 2 days.

2.2.7 Cytotoxicity evaluation

After cell encapsulation, cell cytotoxicity was measured by Live/Dead assay kit (Invitrogen). The cell-laden hydrogels were stained with 0.5 μl of Calcein AM (Green) and 2 μl of ethidium homodimer-1 (Ethd-1, Red) was added in 1ml PBS for 1 hour and washed with PBS for 5mins. The images were taken by a Zeiss LSM 720 Confocal Microscope.

2.2.8 Cell viability analysis

Cell viability assay was performed by alamarBlue™ Cell Viability Reagent (Invitrogen) following the manufacturer's instructions. The absorbance of reacted cell culture media was measured at 570 nm. (n=3)

2.2.9 Real-time PCR analysis:

Cell-laden hydrogels (n=3) were collected on week 3 for gene analysis. Samples were ground and total mRNA was extracted using TRIzol reagent (Life Technologies). mRNA concentration was measured using Infinite 200 PRO NanoQuant Microplate Readers (TECAN). 1 μg of mRNA was reverse

transcribed into cDNA by M-MLV cDNA Synthesis Kit (Enzynomics), according to the manufacturer's instructions. Real-time PCR was performed using SYBR Green PCR master Mix (Enzynomics) and ABI StepOnePlus^{TM} Real-Time RCR system (Applied Biosystems). PCR cycling consisted of 40 cycles of amplification of template DNA with primer annealing at 60°C. All genes were normalized to GAPDH, and the relative gene expression was calculated by $-2^{\Delta\Delta Ct}$ method [63]. Sequences of the primers and probes of *GAPDH*, *COL2*, *SOX 9*, *ACAN*, TNF-α, IL-1β, *MMP13* and *ADAMTS5* are listed in **Table 1**.

2.2.10 Biochemical assay

For biochemical analysis, the cell-laden hydrogels (n=3) were digested using papain solution (Worthington Biomedical) for 16h at 60°C. DNA content was quantified by Quant-iT^{TM} Pico- Green$^{®}$dsDNA assay kit (Invitrogen) according to the manufacturer's instruction. The sGAG accumulation was measured by dimethylmethylene blue (DMMB) spectrophotometric assay at A525. For the total collagen, the hydroxyproline amount was measured by chlormine-T(Sigma Aldrich) colorimetric assay.

2.2.11 Intra-articular injection

Twenty male Balb/c mice (10-week-old) were used for the *in vivo* experiment following the protocols approved by the Institutional Animal

Care and Use Committee (IACUC) of Seoul National University. The mice were randomly divided into 5 groups (*n*=4/group): normal group; PBS group; EGCG 50 μM group; 5% HTG group; 5% HTG-E group. OA induction surgery was performed by anterior cruciate ligament transection (ACLT) [64]. 1-week post-surgery, 5 μL of PBS, 50 μM EGCG, 5% HTG, and 5% HTG-E were injected bilaterally into the knee. After 4 weeks post-injection, the knee joint samples were collected for histological analysis.

2.2.12 Histological analysis

The in vitro hydrogel samples were collected after 3 week-culture, and the mice with intra-articular injection was sacrificed 4 weeks' post-injection. All sample was fixed with 4% PFA solution at 4℃ for 24 hours. Cell-laden hydrogels were embedded in paraffin blocks and sectioned into 5-um-thick slices. For histological staining analysis, the sections were deparaffinized and rehydrated. For H&E staining, sections were stained with hematoxylin (Ricca Chemical Company) for 5min and washed with DI water. then the sections were stained with Eosin-Y (Richard-Allan Scientific) for 1min, followed by multiple washes in DI water. For Safranin-O staining, the sections were rinsed with 0.1% Safranin-O solution (ScholAR Chemistry) for 5 min at room temperature following with dehydrating in a series of gradual ethanol. Type II collagen immunohistochemistry was performed to the sectioning slices. Deparaffinized sections were permeabilized in 1%

bovine serum albumin (BSA, Sigma) solution, and blocking step was performed for 45min with 10% normal goat serum. Anti-Collagen II antibody (ab34712, Abcam) was treated at 4℃ for overnight. After washing with 1% BSA solution, sections were incubated with secondary antibodies (Goat anti-rabbit Alexa-Fluor 546; Life Technologies). the nuclei were stained with DAPI for 5min at room temperature. Imaging was carried out using a fluorescence microscope (EVOS).

Statistical analysis

Statistical analysis: For the statistical studies, GraphPad Prism5 software was used. One-way ANOVA with Tukey posttest, or unpaired two-tailed t-test was performed where applicable. Values of $p < 0.05$ were considered to be statistically significant. Significance level is presented as * $p < 0.05$, ** $p < 0.01$, or *** $p < 0.005$.

2.3 Results

2.3.1 Effect of EGCG on 3-dimensional (3D) encapsulated chondrocyte

EGCG is a polyphenolic bioactive compound isolated from green tea extracts. Many studies have demonstrated the therapeutic effects of EGCG in inflammatory diseases [65, 66]. However, previous studies that provided the therapeutic potential of EGCG were limited to 2-dimensional (2D) cell culture system. In this study, we aimed to fabricate immune modulating hydrogel composed of EGCG and apply this hydrogel in OA disease model for functional recovery. EGCG incorporated hydrogels were fabricated by introducing various amounts of EGCG into the mixture of HA_t and gelatin. We have previously reported the enzymatic kinetic of tyrosinase isolated from *Streptomyces avermitillis* (SA_Ty) for the facilitated tyrosinase based crosslinking of phenolic compounds [61]. Therefore, a combination of HA_t, gelatin and EGCG can be crosslinked to form a hydrogel in a rapid manner by SA_Ty. EGCG-loaded HA_t/Gelatin (HTG-E) hydrogels were examined for their *in vitro* anti-inflammatory responses and applied them in a murine OA model for therapeutic efficacy (**Figure 1**).

In order to optimize EGCG incorporation, we examined the cytotoxicity of EGCG on Chondrocyte in a 3D environment. Chondrocytes that were isolated from porcine articular cartilage and cultured on tissue culture plate for 5 days were utilized for EGCG optimization. chondrocytes ($2x10^7$ cells mL^{-1}, 50 μl/construct) were then mixed with 5% (*w/v*) gelatin solution and

further crosslinked with SA_Ty. Various amounts of EGCG, ranging from 0 μM to 100 μM, were introduced into the gelatin-based hydrogel. After 1 days, Live/Dead staining showed a minor reduction in viability at high concentration of EGCG in 3D culture systems (**Figure 3**A, B). Chondrocytes maintained above 80% viability even with 100 μM EGCG concentration. Interestingly, in monolayer culture system, EGCG (free state in media) concentration above 20 μM showed cytotoxicity (**Figure 2**B). These results suggest that SA_Ty successfully incorporated EGCG into the hydrogel as crosslinker and allowed limited free-stranding EGCG in the medium. We observed that EGCG did not cause serious cytotoxicity to chondrocyte when incorporated into the hydrogel. Therefore, hydrogels incorporated with EGCG can support cell survival for long-term cultivation.

The anti-inflammatory effect of EGCG on monolayer-expanded chondrocytes was reported in several studies [49, 51, 67]. These studies demonstrated that EGCG suppressed the expressions of pro-inflammatory related genes such as *COX2*, *MMP1*, *MMP13*, and *ADMTS5*.[68, 69] our study also showed the same results (Figure 2C). We evaluated whether incorporation of EGCG into the hydrogel would exhibit similar effects on chondrocytes. IL-1β is the main pro-inflammatory cytokine in synovial fluid that causes inflammatory symptoms in OA. For mimicking OA inflammation, chondrocytes were encapsulated in the gelatin-based hydrogel and maintained with culture media containing IL-1β (10 ng mL^{-1}). After 2

days, cells were collected for real-time PCR analysis (Figure 3C). The inflammatory and catabolic genes were all increased after IL-1β stimulation. However, EGCG-dependent down-regulation of inflammatory response genes were observed. In particular, gelatin hydrogel with more than 30 μM EGCG concentration resulted in significant downregulation of *IL-1β, TNF-α*, *MMP13*, and *ADAMTS5*. Gene expression of *IL-1β* and *TNF-α* showed similar levels as that of the control group with the 30 μM EGCG incorporation. In addition, *MMP13* and *ADAMTS5* gene expression levels were downregulated to that of the control group with the 50 μM EGCG incorporation. It demonstrated that EGCG has the ability to regulate inflammation-induced by IL-1β within hydrogels. Based on the real-time PCR analysis, we selected the 50 μM EGCG for further *in vitro* and *in vivo* applications.

2.3.2 Characterization of HTG hydrogel and chondrogenic effects

It is well known that HA promotes chondrogenesis because HA is a major component of the cartilage ECM. However, non-crosslinked HA has limitations such as rapid clearance and low mechanical properties. Several approaches to crosslink HA have been studied. In order to create enzymatically crosslinkable HA, tyramine hydrochloride was conjugated onto HA backbone via EDC/NHS chemistry. Then, tyramine-conjugated HA(HA_t) was mixed with gelatin, followed by crosslinking through

tyrosinase-mediated crosslinking reaction. This enzyme-based crosslinking is relatively rapid and can be applied as an injectable system. In order to incorporate HA into the crosslinked hydrogel, 2% or 5% (*w/v*) HA_t was mixed with 5% (*w/v*) gelatin in the presence of 6 μM SA_Ty. We used gelatin without HA_t as control hydrogel. Since gelatin itself has a reactive tyrosine group, HA/Gelatin hydrogel without HA_t showed brown color (0% HTG), as a result of SA_Ty crosslinking. Additionally, HA/Gelatin (HTG) hydrogels showed gradually darker brown color as HA_t concentration increased (**Figure 4**A). The rheological properties of HTG hydrogels were evaluated by rheometer (Figure 4B). Both amplitude and frequency sweep exhibited HA_t concentration-dependent manner. 5% HTG hydrogel showed the highest storage modulus (G'). Swelling analysis showed reciprocal trends as the storage modulus (Figure 4C). 5% HTG hydrogel showed the lowest degree of water uptake. Both rheology and swelling test results confirmed the highest crosslinking degree and strength property of 5% HTG hydrogel.

We also measured the enzymatic degradation of hydrogels (Figure 4D). As the hydrogel was composed of the HA and gelatin, hyaluronidase and collagenase (10 U mL^{-1}) were treated respectively. After 2 weeks of hyaluronidase degradation, all groups were degraded completely. However, in the collagenase degradation test, all groups except 0% HTG hydrogel remained after 2 weeks. This demonstrated that the gelatin-related

crosslinking is rapidly degraded by enzymes and the HA_t-related crosslinking is strong so that the degradation is not easy.

2.3.3 Evaluation of chondrogenic effects of HTG hydrogel

To evaluate whether the HTG hydrogel can be applied in cartilage tissue regeneration, porcine articular chondrocytes ($2x10^7$ cells mL^{-1}) were encapsulated in HTG hydrogels and evaluated the tissue constructs after 3 weeks of culture. After 3 weeks of culture, constructs were harvested and biochemical analysis was performed (**Figure 5**A). We quantified the sulfated GAG amount by DMMD assay and quantified collagen content by hydroxyproline content analysis. All the measurements were normalized to the respective DNA amount. 0% HTG hydrogel at day 1 served as control. As the HA_t content was increased within the HTG hydrogel, overall collagen and GAG contents within the hydrogel were increased (Figure 5A). Additionally, 5% HTG exhibited the highest level of collagen compared to 0% and 2% HTG groups. Histological analysis confirmed with biochemical analysis (Figure 5B). H&E staining showed reduced cellularity in 5% HTG compared to the 2% HTG, confirming chondrocytes within 5% HTG produced more ECM. Additionally, Safranin-O staining showed the intense red-stained area in 5% HTG hydrogel compared to the 2% HTG hydrogel as a result of increased in GAG accumulation within the hydrogel. Finally, type II collagen immunostaining showed that the cells in 5% HTG hydrogel

retained circular morphology and the type II collagen were retained within the cellular periphery. Cells in 2% HTG hydrogels were spindle-shaped and elongated. Furthermore, counterstaining with DAPI confirmed the increased cellularity within 2% HGT hydrogel. Interestingly, 0% HTG (HA/Gelatin hydrogel without HA_t), resulted in complete degradation within 5 days of culture (data not shown). This may be due to the reduced crosslinking of the hydrogel as a result of 0% HA_t along with the active cellular reorganization and degradation.

We also measured the cartilage-specific gene expression levels of the cell-laden HTG hydrogels. Real-time PCR was used to compare the level of chondrogenic genes such as *type II collagen* (*COL2*), *Sex-determining region Y box 9* (*SOX9*) and *aggrecan* (*ACAN*) at week 3. The data is shown in **Figure 5**C. All gene expressions were normalized to 0% HTG hydrogel collected on day 1. All genes showed similar trends compared to biochemical analysis. Gene levels in 5% HTG hydrogel group were higher than those in other groups. From this work, we confirmed that HTG composite hydrogel can promote chondrogenesis, with the higher chondrogenic property as HA content increases.

2.3.4 Synergetic effect of HTG hydrogel combined with EGCG(HTG-E)

To confirm the synergetic effect of EGCG and HTG hydrogel, we encapsulated chondrocytes in 5% HTG hydrogels or 5% HTG-E hydrogels

(with 50 μM EGCG) and treated with 10 ng mL^{-1} of IL-1β for 3 weeks to mimic the inflammatory environment of OA. EGCG was mixed with hydrogel precursor to evaluate its anti-inflammatory effect. **Figure 6**A showed histological analysis 5% HTG and 5% HTG-E cultured for 3 weeks. Safranin-O staining indicated more GAG accumulation in 5% HTG-E group than 5% HTG group. Col II immunostaining results showed type II collagen production in the pericellular area in both groups. However, the expression of Col II protein was more prominent in 5% HTG-E hydrogel compared to EGCG absent group. The presence of IL-1β leads to cartilage ECM degradation in OA knee joint. This might lead to reduced syntheses of ECM like GAG and Col II by the chondrocyte in 5% HTG, which were preserved in EGCG-containing hydrogel. EGCG not only has an anti-inflammatory effect as shown in Figure 3C, but also chondroprotective effect in long-term inflammation.

The chondrogenic and inflammatory gene expressions were evaluated by real-time PCR (Figure 6B and C). As anticipated, inflammatory gene expressions were down-regulated in EGCG-containing group, confirming EGCG's inflammatory effects in the HTG hydrogel *in vitro*. The chondrogenic genes were up-regulated in 5% HTG-E group, which is consistent with the histological analysis results.

2.3.5 In vivo OA treatment with HTG-E hydrogel in mouse OA model

EGCG with HTG hydrogel was intra-articularly administered to OA-induced mice to determine the effect on OA progression. After 1-week OA induction surgery, PBS, 50 μM EGCG, 5% HTG, and 5% HTG-E were each injected into the knee. The knee joints were collected for histological analysis after 4 weeks (**Figure 7**). Safranin-O staining and type II collagen immunostaining demonstrated that PBS injection group had very severely eroded and thinned cartilage and notable GAG and type II collagen loss. Although less severe than PBS group, 50 μM EGCG and 5% HTG hydrogel groups showed some degree of cartilage ECM loss and surface erosion compared to normal cartilage. When EGCG and the hydrogel were injected together as in the 5% HTG-E group, GAG and collagen contents up to par with that of native cartilage. Furthermore, in 5% HTG-E, a cartilage surface, and thickness were completely intact, showing no signs of wear and tear. These results indicate that treatment with the combination of EGCG and HTG hydrogel retarded the progression of OA, while each of components alone also had mild beneficial effects.

2.4 Discussion

OA is a type of cartilage degenerative disease that results from the breakdown of joint cartilage and underlying bone. With OA progression, ECM components in cartilage degrade gradually as pro-inflammatory environment promotes ECM deconstruction. IL-1β is one of the pro-inflammation cytokines in the osteoarthritic joint synovium. Therefore, the sequester of pro-inflammation cytokine-like IL-1β may have significant therapeutic benefits in preventing OA progression and restoring tissue homeostasis.

In recent years, several biomaterials approaches have attempted to modulate the immune response in a manner that can more effectively promote regeneration at the site of injury [70-72]. These include the modulation in biomaterials surface chemistry to influence the protein adsorption. Selectivity of adsorbed proteins on biomaterials may mediate the interactions with immune cells and that may ultimately control the immune cell activation. In addition, a strategy that employs chemical crosslinking of natural ECM has been employed to increase the stability of naturally derived biomaterials. Interestingly, recent evidence supports the notion that types of crosslinking strategy employed may impact the immune response. Recently, Brown *el. al*., showed that acellular ECM without chemical modification often result in an M2 macrophage response while carbodiimide (CDI) cross-linked scaffolds induce M1 response [73]. In our

study, we employed tyrosinase induced crosslinking of tyramine conjugated HA and gelatin. Tyrosinase activity is involved in our body as a natural crosslinking process that induces accumulation of melanin. Our previous report has suggested that SA_Ty based crosslinking of natural ECM (such as gelatin) allow a minimal immune response. Furthermore, SA_Ty based crosslinking allowed very stable hydrogel system that can withstand collagenase/hyaluronidase induced degradation (Figure 4D). In addition, SA_Ty based crosslinking system not only allowed the hydrogel formation but also induced the stable adhesion of the HTG hydrogel on to the cartilage surface. Our previous report demonstrated the high degree of adhesion of gelatin-based hydrogels onto the various tissues [61, 62]. As the hydrogel can imbibe a large amount of water, our HTG hydrogel may have contributed to the sequestration of inflammatory cytokines upon injection into OA animal model, creating a local anti-inflammatory microenvironment that influenced the regeneration.

In this study, we also incorporated EGCG and hyaluronic acid into our hydrogel platform to the added benefit of controlling OA inflammatory microenvironment. EGCG is a polyphenolic compound that has been shown to possess anti-inflammatory activity with a potential for anti-arthritic effects [50]. Due to its phenolic functional groups, it can scavenge radical oxygen species, and it has shown to reduce the IL-1β induced inflammatory response. Indeed, we have confirmed that the chondrocytes under

pathophysiological conditions exhibited significant down-regulation of gene expression levels in the inflammatory targets included IL-1β when treated with EGCG (Figure 3C). However, we noticed that EGCG over 20 μM resulted in cellular cytotoxicity in monolayer culture system (Figure 2). However, when we performed the viability of cells within the HTG-E hydrogel, we were able to load up to 100 μM of EGCG on to the HTG-E hydrogel with minimal cytotoxicity. This could be the result of covalent conjugation of EGCG (with its phenolic functional groups) onto the HA_t or gelatin, resulting in covalent incorporation of EGCG on to the hydrogel. Indeed, rheological and mechanical properties comparing 5% HTG vs 5% HTG-E showed that increased crosslinking density along with increased mechanical properties of 5% HTG-E hydrogel due to the additional crosslinking as result of EGCG incorporation. As the significant amount of EGCG was covalently incorporated on to the hydrogel, we hypothesize that EGCG induced cytotoxicity was minimized in the 3D hydrogel environment compared to the 2D culture platform. In addition, our study demonstrates the added benefit of incorporating EGCG on to the tyrosinase-induced crosslinking hydrogel for effective anti-inflammatory control via EGCG.

HA is one of the most abundant glycosaminoglycans (GAGs) in cartilage ECM. In addition, HA in synovial fluid provides lubrication, along with the lubricin [74]. Even though the therapeutic effect of intra-articular

injection of HA to OA joint has been reported the HA injection provide only a transient treatment option for OA patient as it is not directly involved in the cartilage regeneration and has relatively rapid clearance [75]. As a way to provide cell friendly environment for the cartilage and provide a lubricating component to the injected hydrogel, we introduced tyramine-conjugated HA.

When chondrocytes were encapsulated in a HTG hydrogel with various amount of HA_t concentration, we observed the HA_t dependent ECM accumulation. Previously, crosslinked HA-based hydrogel has been extensively studied for cartilage tissue engineering for past decades. Levett *et al*. and Moulisova *et al*. have reported crosslinked HA-based hydrogels promoted the GAG and collagen II accumulation [76, 77]. In accordance with the previous study, we also demonstrated that higher the HA content more ECM were accumulated within the hydrogel [78]. Histological analysis and immunostaining confirmed the increased ECM accumulation in 5% HTG when compared to the 2% HTG constructs. Interestingly, cellular phenotypes and organization of type II collagen were drastically different when we compared the tissue sections of 5% HTG versus 2% HTG (Figure 5B). In 5% HTG hydrogel, cells maintained circular phenotype and type II collagen were typically located around the cells. However, within 2% HTG hydrogel, cells appeared to be stretched and collagens orientation were distinct. This may be due to the reduced HA content in 2% HGT hydrogel

that may allow increased cellular adhesion to the gelatin component within the hydrogel. Furthermore, this may be due to the reduced amount of HA_t that may contribute to the larger crosslinking density, which may have contributed to the cell-induced contraction of the hydrogel construct. Cytoskeletal tension within chondrocytes are responsible for dedifferentiation and retaining of fibroblast-like morphology. We observed higher cellularity and reduced ECM accumulation in 2% HTG hydrogel compared to the 5% HTG. Even though cellular adhesion to biomaterials is beneficial for cell survival, hyper adhesion to the biomaterials may result in reduced cellular bioactivity. Indeed, mesenchymal stem cells under reduced cytoskeletal tension, such as RhoA, can efficiently undergo chondrogenesis. This is one of the primary reason why the chondrocytes cultured in the 2D platform isn't able to synthesize GAGs and down regulate the chondrocyte-specific genes such as *COL2*, *SOX9*, and *ACAN* [79]. In contrast, 3D cultivation of chondrocytes can support the re-differentiation by keeping the cell in a spherical shape [80]. Our result showed that chondrocytes in 5% HTG hydrogel retained more circular cellular shape due to the added crosslinking by HA_t.

Based on EGCG induced anti-inflammation effects and optimized HA_t concentration to induce optimal ECM production, we incorporated EGCG into 5% HTG hydrogel for the treatment of OA. Prior to applying into the OA animal model, cell-laden hydrogels with or without EGCG were

cultured in IL-1β contained media for 3 weeks to investigate whether the HTG-E hydrogel could resist to OA environment. Our findings suggested that 5% HTG-E hydrogel provide a protective effect from the OA-like inflammatory environment. The accumulation of ECM was detected in the 5% HTG-E group rather than in the 5% HTG group, and the inflammatory gene was down-regulated through the 3-week culture. On the other hand, the cartilage forming gene was upregulated in the 5% HTG-E group. *In vivo* evaluation of intra-articular injection of 5% HTG-E showed progressive results in OA treatment. OA was initiated by anterior cruciate ligament transection (ACLT), and 5 μl of hydrogels (5% HTG with or without EGCG) were injected. All the tissues were evaluated after 4 weeks. In the PBS injection control group, the cartilage was completely damaged. In the 50 μM EGCG and 5% HTG hydrogel injection groups, the cartilage was thinned but out. However, articular cartilage tissues were not completely eroded. These results indicated that the injection of 50μM EGCG or 5% HTG hydrogel alone provided the therapeutic benefit to OA. Our study is in accordance with previous studies that reported the beneficial effects of EGCG on the OA model. Leong *et al*., have reported that the daily intraperitoneal injection of EGCG to OA induced mice exhibited relief of OA-related pain and reduction of proinflammatory cytokine levels [81]. In another study by Natarajan *et al*., intra-articular injections of polyphenols such as EGCG and Tannic acid showed the resistant to degradation despite

ongoing inflammation [82]. This study was performed with EGCG and Tananic acid injection every 3days for the significant outcome. Although intra-articular injection of drugs provides one option for drug delivery in a joint, the rapid clearance of the injected drug limits therapeutic efficacy of the intra-articular injection.

In our study, we have allowed crosslinking of EGCG into our hydrogel. Our HTG-E hydrogel can be retained in the joint for a long time. Due to the adhesive property of HTG-E hydrogel, anti-inflammatory EGCG components can be retained within the surface of articular cartilage [62]. A recent study by Emoto *et al* demonstrated the *in situ* cross-linkable HA hydrogels. This HA-based hydrogel was able to sustain the release of anti-cancer drug within several days [83]. For the combination treatment of EGCG and HA-based hydrogel, 5% HTG hydrogel injection group showed hyaline cartilage similar to that of the normal control group. We did not detect any wear and ECM loss. The injected HTG-E hydrogel may have formed a thin layer on the cartilage surface to protect cartilage from physical abrasion. Furthermore, EGCG component within HTG-E hydrogel may provide an anti-inflammatory microenvironment that allowed maintenance of articular cartilage. Based on our initial finding, we believe that this inflammatory modulating hydrogel can be applied in various tissue augment applications.

Conclusion

In summary, we demonstrated that HTG hydrogel promotes ECM accumulation and EGCG-loaded hybrid hydrogel has anti-inflammatory and chondroprotective capacities in IL-1β induced OA environment. Injection of the EGCG-loaded hybrid hydrogel to surgically induced OA knee joint can protect cartilage from inflammation and 'wear and tear'. Therefore, we believe that this EGCG-loaded injectable HTG hydrogel is promising in tissue engineering and has great potential for clinical recovery of OA.

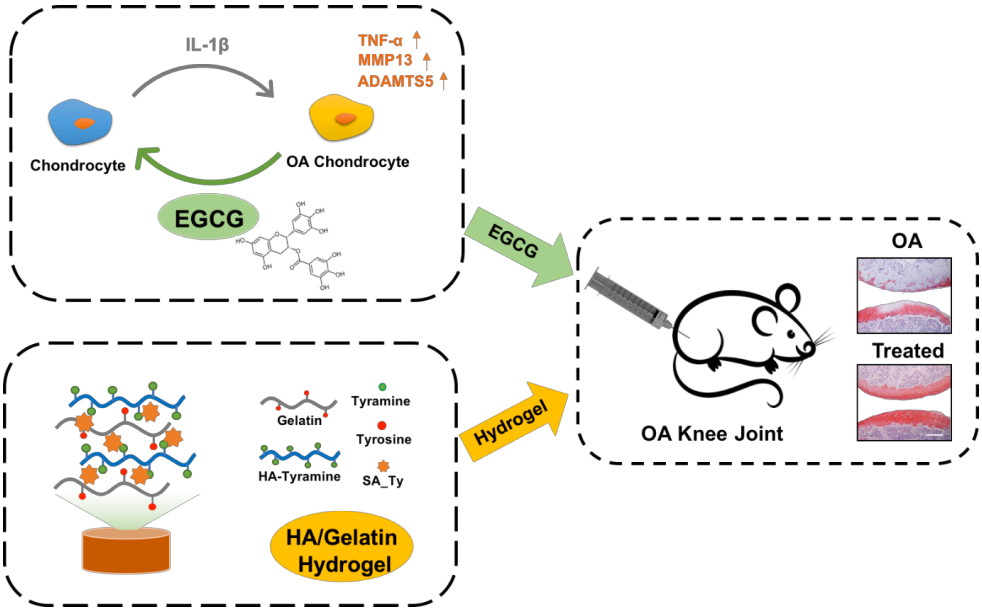

Figure 1. Schematic illustration of evaluating the inflammation modulation HA hydrogel.

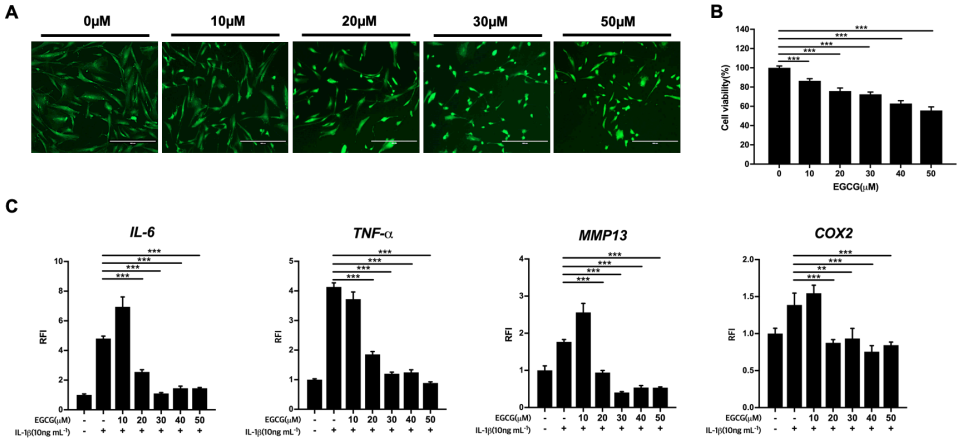

Figure 2. EGCG effect on monolayer cultured chondrocytes in inflammatory-induced environment. (A) Live/Dead assay for dose-dependent EGCG treatment; Live cell was stained with by Calcein AM (green) and dead cell was washed out when staining. Scale bar=400μm. (B) Cell viability assay was measured by AlamarBlue™ reagent. (C) Real-time PCR analysis of inflammatory genes of *IL-6*, *TNF-α*, *MMP13* and *COX2* genes (*n*=3), RFI=Related Fold Induction.

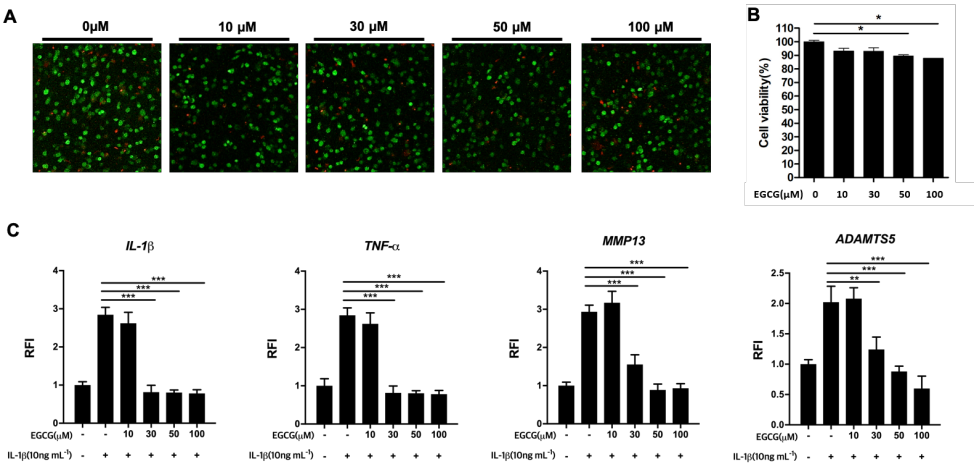

Figure 3. EGCG effect on 3D encapsulated chondrocytes in OA-like environment. (A) Live/Dead assay for dose-dependent EGCG treatment; Live cell was stained with by Calcein AM (green) and dead cell was stained by Ethdium homodimr-1 (red). Scale bar=100μm. (B) Cell viability assay was measured by AlamarBlue™ reagent. (C) Real-time PCR analysis inflammatory genes of *IL-1β*, *TNF-α*, *MMP13* and *ADAMTS5* genes (*n*=3).

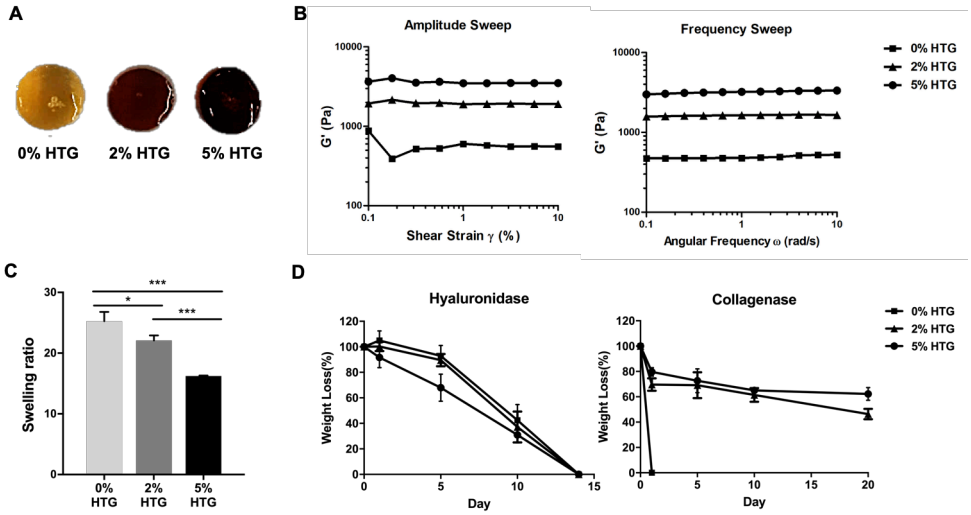

Figure 4. Characterization of HTG hydrogel. (A) Gross images of 0%, 2% and 5% HTG hydrogels. The final concentration of each hydrogel was composed of 0%, 2%, 5% (*w/v*) HA_t and 5% (*w/v*) gelatin. (B) Rheological analysis of HTG hydrogels. G'=storage modulus. (C) Swelling ratio (*n*=3). (D) Enzymatic degradation of HTG hydrogels (10 unit of each enzyme; *n*=3).

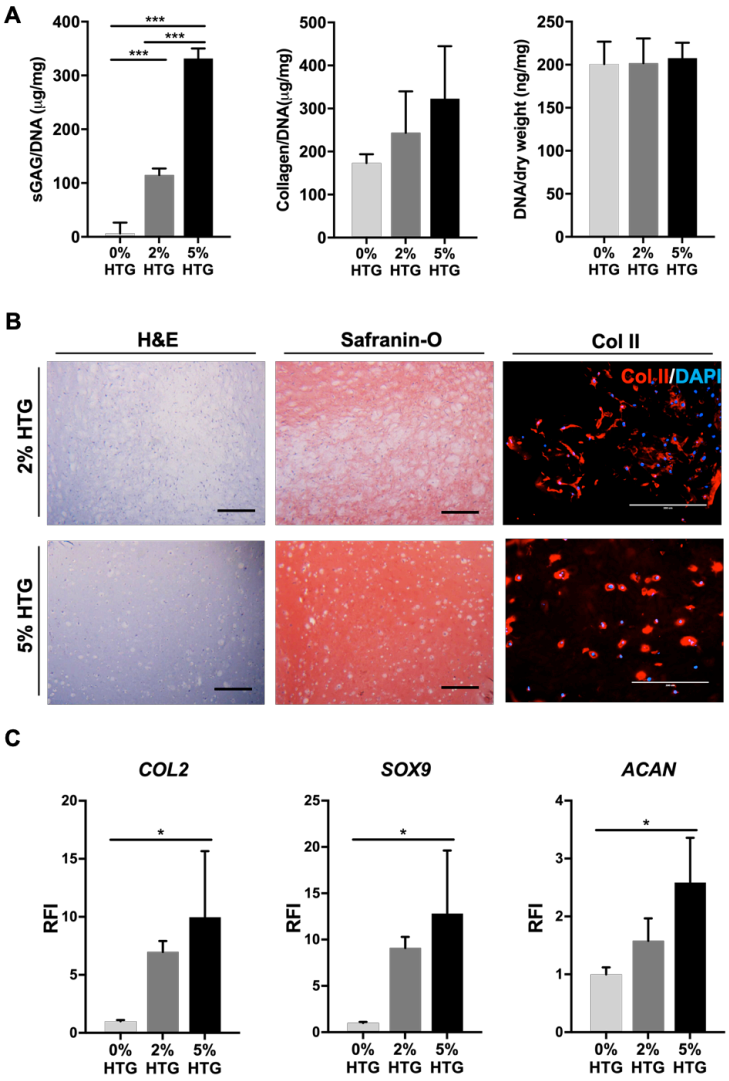

Figure 5. Comparison of the chondrogenic properties of 2% and 5% HTG hydrogel through *in vitro* cell encapsulation for 3weeks. (A) Biochemical analysis of sGAG, collagen and DNA content (*n*=3). 0% HTG hydrogel collected at day 1 was served as control. (B) Histological analysis of H&E, Safranin-O and Col II immunohistochemistry. Scale bar= 200 μm (C) Real-time PCR results of *COL2*, *SOX9*, and *ACAN* genes (*n*=3). 0% HTG hydrogel collected at day 1 was served as control.

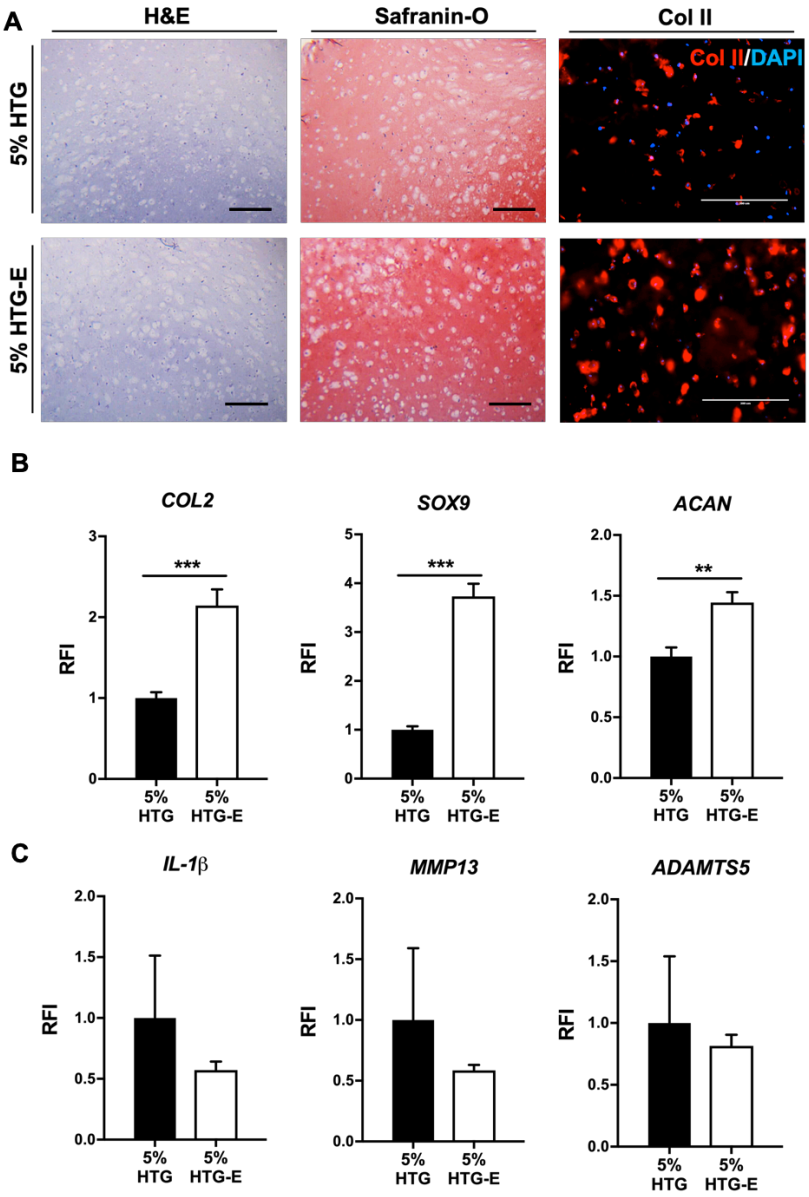

Figure 6. *In vitro* evaluation of 5% HTG and 5% HTG-E hydrogel in OA mimetic environment. (A) Histological analysis of H&E, Safranin-O and Col II immunohistochemistry. Scale bar= 200μm (B) Real-time PCR results of *IL-1β*, *MMP13*, and *ADAMTS5*(*n*=3). (C) Real-time PCR results of *COL2*, *SOX9*, and *ACAN* (*n*=3).

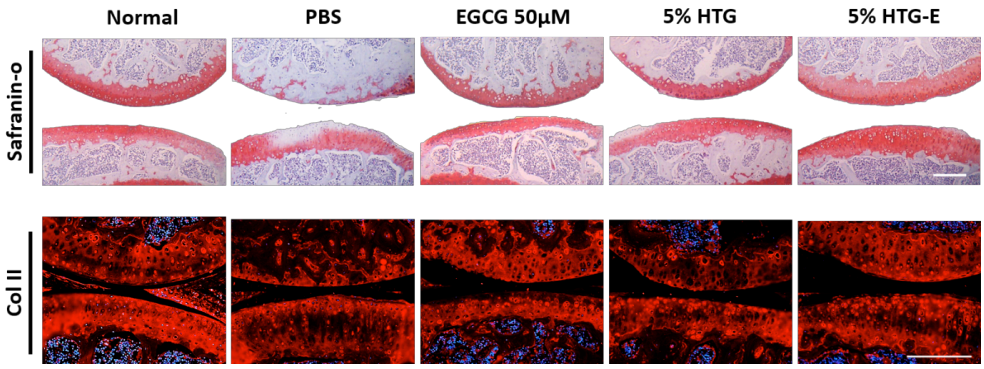

Figure 7. Intra-articular injection of 5% HTG-E hydrogel in OA mouse joint. Histological analysis by Safranin-O and Col II staining. Scale bar=200 μm

Table 1. Primer list.

		Primer sequences (5'-3')
GAPDH	Forward	TCA GCT GCT GGG GAG TCA CA
	Reverse	CCT AAG CCC CTC CCC TTC TT
IL-1β	Forward	TCT CTG GAC CCA AAG GAG GG
	Reverse	ACG GCT GCT TTC ACG GGT GA
TNF-a	Forward	AGC TGG TGG TGC CGA CAG AT
	Reverse	TGC GAT GCG GCT GAT GGT GT
MMP13	Forward	CCT GTG CTC CTG CCA TTT GG
	Reverse	GAA TGG GCA GCT CCA TGG CT
ADAMTS5	Forward	CGC TCC CTG GCT GTC TTT GA
	Reverse	TCA AAG TGT AGC GCG CGT GC
COL2	Forward	TAG AGA GGT TTC CTG GGC CG
	Reverse	AGG AGG ACG CTG GAA CAG AG
SOX9	Forward	CAG TCC CAG CGA ACG CAC AT
	Reverse	TGC TGC TGC TGC TCG CTG TA
ACAN	Forward	CTG CCC CGA AAC ATC ACC GA
	Reverse	GTA AAG GGC TCC TCA GGC TC

References

1. Nelson, A.E., *Osteoarthritis year in review 2017: clinical.* Osteoarthritis Cartilage, 2018. **26**(3): p. 319-325.
2. Schmidt, T.A., et al., *Boundary lubrication of articular cartilage: role of synovial fluid constituents.* Arthritis Rheum, 2007. **56**(3): p. 882-91.
3. Martel-Pelletier, J., et al., *Cartilage in normal and osteoarthritis conditions.* Best Pract Res Clin Rheumatol, 2008. **22**(2): p. 351-84.
4. Otterness, I.G., et al., *An analysis of 14 molecular markers for monitoring osteoarthritis: segregation of the markers into clusters and distinguishing osteoarthritis at baseline.* Osteoarthritis Cartilage, 2000. **8**(3): p. 180-5.
5. Blasioli, D.J. and D.L. Kaplan, *The roles of catabolic factors in the development of osteoarthritis.* Tissue Eng Part B Rev, 2014. **20**(4): p. 355-63.
6. Sophia Fox, A.J., A. Bedi, and S.A. Rodeo, *The basic science of articular cartilage: structure, composition, and function.* Sports Health, 2009. **1**(6): p. 461-8.
7. Fahy, N., et al., *Immune modulation to improve tissue engineering outcomes for cartilage repair in the osteoarthritic joint.* Tissue Eng Part B Rev, 2015. **21**(1): p. 55-66.
8. Rigoglou, S. and A.G. Papavassiliou, *The NF-kappaB signalling pathway in osteoarthritis.* Int J Biochem Cell Biol, 2013. **45**(11): p. 2580-4.
9. Zeng, G.Q., et al., *High MMP-1, MMP-2, and MMP-9 protein levels in osteoarthritis.* Genet Mol Res, 2015. **14**(4): p. 14811-22.
10. Yang, C.Y., A. Chanalaris, and L. Troeberg, *ADAMTS and ADAM metalloproteinases in osteoarthritis - looking beyond the 'usual suspects'.* Osteoarthritis Cartilage, 2017. **25**(7): p. 1000-1009.
11. Ulivi, V., et al., *p38/NF-kB-dependent expression of COX-2 during differentiation and inflammatory response of chondrocytes.* J Cell Biochem, 2008. **104**(4): p. 1393-406.
12. Varga, Z., S.R.A. Sabzwari, and V. Vargova, *Cardiovascular Risk of Nonsteroidal Anti-Inflammatory Drugs: An Under-Recognized Public Health Issue.* Cureus, 2017. **9**(4): p. e1144.
13. Rollason, V., et al., *Prediction by pharmacogenetics of safety and efficacy of non-steroidal anti- inflammatory drugs: a review.* Curr Drug Metab, 2014. **15**(3): p. 326-43.
14. Cho, H., et al., *Study of osteoarthritis treatment with anti-inflammatory drugs: cyclooxygenase-2 inhibitor and steroids.* Biomed Res Int, 2015. **2015**: p. 595273.
15. Nasr, M., *In vitro and in vivo evaluation of proniosomes containing celecoxib for oral administration.* AAPS PharmSciTech, 2010. **11**(1):

p. 85-9.
16. Makela, A.L., M. Lempiainen, and H. Ylijoki, *Ibuprofen levels in serum and synovial fluid.* Scand J Rheumatol Suppl, 1981. **39**: p. 15-7.
17. Netter, P., B. Bannwarth, and M.J. Royer-Morrot, *Recent findings on the pharmacokinetics of non-steroidal anti-inflammatory drugs in synovial fluid.* Clin Pharmacokinet, 1989. **17**(3): p. 145-62.
18. Evans, C.H., V.B. Kraus, and L.A. Setton, *Progress in intra-articular therapy.* Nat Rev Rheumatol, 2014. **10**(1): p. 11-22.
19. Cooper, C., et al., *Use of Intraarticular Hyaluronic Acid in the Management of Knee Osteoarthritis in Clinical Practice.* Arthritis Care Res (Hoboken), 2017. **69**(9): p. 1287-1296.
20. Bellamy, N., et al., *Viscosupplementation for the treatment of osteoarthritis of the knee.* Cochrane Database Syst Rev, 2006(2): p. CD005321.
21. Colen, S., et al., *Hyaluronic acid in the treatment of knee osteoarthritis: a systematic review and meta-analysis with emphasis on the efficacy of different products.* BioDrugs, 2012. **26**(4): p. 257-68.
22. Wang, Y., et al., *Effects of Hylan G-F 20 supplementation on cartilage preservation detected by magnetic resonance imaging in osteoarthritis of the knee: a two-year single-blind clinical trial.* BMC Musculoskelet Disord, 2011. **12**: p. 195.
23. Fraser, J.R., T.C. Laurent, and U.B. Laurent, *Hyaluronan: its nature, distribution, functions and turnover.* J Intern Med, 1997. **242**(1): p. 27-33.
24. Burke, J., et al., *Therapeutic potential of mesenchymal stem cell based therapy for osteoarthritis.* Clin Transl Med, 2016. **5**(1): p. 27.
25. Welch, T., B. Mandelbaum, and M. Tom, *Autologous Chondrocyte Implantation: Past, Present, and Future.* Sports Med Arthrosc Rev, 2016. **24**(2): p. 85-91.
26. Robinson, S., et al., *Assessment of Cartilage Growth After Biopsy of Osteochondral Loose Bodies in Adolescent Knees for Use in Autologous Chondrocyte Implantation.* J Pediatr Orthop, 2018.
27. Freitag, J., et al., *Mesenchymal stem cell therapy in the treatment of osteoarthritis: reparative pathways, safety and efficacy - a review.* BMC Musculoskelet Disord, 2016. **17**: p. 230.
28. Park, Y.B., et al., *Cartilage Regeneration in Osteoarthritic Patients by a Composite of Allogeneic Umbilical Cord Blood-Derived Mesenchymal Stem Cells and Hyaluronate Hydrogel: Results from a Clinical Trial for Safety and Proof-of-Concept with 7 Years of Extended Follow-Up.* Stem Cells Transl Med, 2017. **6**(2): p. 613-621.
29. Jo, C.H., et al., *Intra-articular injection of mesenchymal stem cells for the treatment of osteoarthritis of the knee: a proof-of-concept clinical trial.* Stem Cells, 2014. **32**(5): p. 1254-66.

30. Wyles, C.C., et al., *Mesenchymal stem cell therapy for osteoarthritis: current perspectives.* Stem Cells Cloning, 2015. **8**: p. 117-24.
31. Toh, W.S., et al., *MSC exosome as a cell-free MSC therapy for cartilage regeneration: Implications for osteoarthritis treatment.* Semin Cell Dev Biol, 2017. **67**: p. 56-64.
32. Murphy, M.B., K. Moncivais, and A.I. Caplan, *Mesenchymal stem cells: environmentally responsive therapeutics for regenerative medicine.* Exp Mol Med, 2013. **45**: p. e54.
33. Lim, C.L., et al., *Immunogenicity and immunomodulatory effects of the human chondrocytes, hChonJ.* BMC Musculoskelet Disord, 2017. **18**(1): p. 199.
34. Lu, H.T., et al., *Injectable hyaluronic-acid-doxycycline hydrogel therapy in experimental rabbit osteoarthritis.* BMC Vet Res, 2013. **9**: p. 68.
35. Miyazaki, T., C. Yomota, and S. Okada, *Development and release characterization of hyaluronan-doxycycline gels based on metal coordination.* J Control Release, 2001. **76**(3): p. 337-47.
36. Petit, A., et al., *Sustained intra-articular release of celecoxib from in situ forming gels made of acetyl-capped PCLA-PEG-PCLA triblock copolymers in horses.* Biomaterials, 2015. **53**: p. 426-36.
37. Bedouet, L., et al., *Synthesis of hydrophilic intra-articular microspheres conjugated to ibuprofen and evaluation of anti-inflammatory activity on articular explants.* Int J Pharm, 2014. **459**(1-2): p. 51-61.
38. Zhang, Z., et al., *Intra-Articular Injection of Cross-Linked Hyaluronic Acid-Dexamethasone Hydrogel Attenuates Osteoarthritis: An Experimental Study in a Rat Model of Osteoarthritis.* Int J Mol Sci, 2016. **17**(4): p. 411.
39. Goldring, M.B., *The role of the chondrocyte in osteoarthritis.* Arthritis Rheum, 2000. **43**(9): p. 1916-26.
40. Daheshia, M. and J.Q. Yao, *The interleukin 1beta pathway in the pathogenesis of osteoarthritis.* J Rheumatol, 2008. **35**(12): p. 2306-12.
41. Wojdasiewicz, P., L.A. Poniatowski, and D. Szukiewicz, *The role of inflammatory and anti-inflammatory cytokines in the pathogenesis of osteoarthritis.* Mediators Inflamm, 2014. **2014**: p. 561459.
42. Stove, J., et al., *Interleukin-1beta induces different gene expression of stromelysin, aggrecan and tumor-necrosis-factor-stimulated gene 6 in human osteoarthritic chondrocytes in vitro.* Pathobiology, 2000. **68**(3): p. 144-9.
43. Hwang, J.T., et al., *Apoptotic effect of EGCG in HT-29 colon cancer cells via AMPK signal pathway.* Cancer Lett, 2007. **247**(1): p. 115-21.
44. Yang, J., et al., *EGCG attenuates high glucose-induced endothelial cell inflammation by suppression of PKC and NF-kappaB signaling in*

human umbilical vein endothelial cells. Life Sci, 2013. **92**(10): p. 589-97.
45. Valcic, S., et al., *Antioxidant chemistry of green tea catechins. Identification of products of the reaction of (-)-epigallocatechin gallate with peroxyl radicals.* Chem Res Toxicol, 1999. **12**(4): p. 382-6.
46. Nakamuta, M., et al., *Epigallocatechin-3-gallate, a polyphenol component of green tea, suppresses both collagen production and collagenase activity in hepatic stellate cells.* Int J Mol Med, 2005. **16**(4): p. 677-81.
47. Zhong, Y., et al., *Anti-inflammatory activity of lipophilic epigallocatechin gallate (EGCG) derivatives in LPS-stimulated murine macrophages.* Food Chem, 2012. **134**(2): p. 742-8.
48. Jung, I.H., et al., *Anti-inflammatory effect of (-)-epigallocatechin-3-gallate on Porphyromonas gingivalis lipopolysaccharide-stimulated fibroblasts and stem cells derived from human periodontal ligament.* J Periodontal Implant Sci, 2012. **42**(6): p. 185-95.
49. Akhtar, N. and T.M. Haqqi, *Epigallocatechin-3-gallate suppresses the global interleukin-1beta-induced inflammatory response in human chondrocytes.* Arthritis Res Ther, 2011. **13**(3): p. R93.
50. Singh, R., N. Akhtar, and T.M. Haqqi, *Green tea polyphenol epigallocatechin-3-gallate: inflammation and arthritis. [corrected].* Life Sci, 2010. **86**(25-26): p. 907-18.
51. Ahmed, S., *Green Tea Polyphenol Epigallocatechin-3-gallate (EGCG) Differentially Inhibits Interleukin-1 -Induced Expression of Matrix Metalloproteinase-1 and -13 in Human Chondrocytes.* Journal of Pharmacology and Experimental Therapeutics, 2003. **308**(2): p. 767-773.
52. Rasheed, Z., et al., *Green tea polyphenol epigallocatechin-3-gallate inhibits advanced glycation end product-induced expression of tumor necrosis factor-alpha and matrix metalloproteinase-13 in human chondrocytes.* Arthritis Res Ther, 2009. **11**(3): p. R71.
53. Ahmed, S., et al., *Green tea polyphenol epigallocatechin-3-gallate inhibits the IL-1 beta-induced activity and expression of cyclooxygenase-2 and nitric oxide synthase-2 in human chondrocytes.* Free Radic Biol Med, 2002. **33**(8): p. 1097-105.
54. Singh, R., et al., *Epigallocatechin-3-gallate inhibits interleukin-1beta-induced expression of nitric oxide synthase and production of nitric oxide in human chondrocytes: suppression of nuclear factor kappaB activation by degradation of the inhibitor of nuclear factor kappaB.* Arthritis Rheum, 2002. **46**(8): p. 2079-86.
55. McAlindon, T.E., et al., *OARSI guidelines for the non-surgical management of knee osteoarthritis.* Osteoarthritis Cartilage, 2014. **22**(3): p. 363-88.
56. Rey-Rico, A., H. Madry, and M. Cucchiarini, *Hydrogel-Based*

Controlled Delivery Systems for Articular Cartilage Repair. Biomed Res Int, 2016. **2016**: p. 1215263.
57. Brown, T.J., U.B. Laurent, and J.R. Fraser, *Turnover of hyaluronan in synovial joints: elimination of labelled hyaluronan from the knee joint of the rabbit.* Exp Physiol, 1991. **76**(1): p. 125-34.
58. Larsen, N.E., et al., *Clearance kinetics of a hylan-based viscosupplement after intra-articular and intravenous administration in animal models.* J Biomed Mater Res B Appl Biomater, 2012. **100**(2): p. 457-62.
59. Camci-Unal, G., et al., *Hydrogel surfaces to promote attachment and spreading of endothelial progenitor cells.* J Tissue Eng Regen Med, 2013. **7**(5): p. 337-47.
60. Taddei, P., et al., *Silk Fibroin/Gelatin Blend Films Crosslinked with Enzymes for Biomedical Applications.* Macromolecular Bioscience, 2013. **13**(11): p. 1492-1510.
61. Kim, S.H., et al., *Enzyme-mediated tissue adhesive hydrogels for meniscus repair.* Int J Biol Macromol, 2018. **110**: p. 479-487.
62. Kim, S.H., et al., *Tissue adhesive, rapid forming, and sprayable ECM hydrogel via recombinant tyrosinase crosslinking.* Biomaterials, 2018. **178**: p. 401-412.
63. Livak, K.J. and T.D. Schmittgen, *Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method.* Methods, 2001. **25**(4): p. 402-8.
64. Kamekura, S., et al., *Osteoarthritis development in novel experimental mouse models induced by knee joint instability.* Osteoarthritis Cartilage, 2005. **13**(7): p. 632-41.
65. Tipoe, G.L., et al., *Epigallocatechin-3-gallate (EGCG) reduces liver inflammation, oxidative stress and fibrosis in carbon tetrachloride (CCl4)-induced liver injury in mice.* Toxicology, 2010. **273**(1-3): p. 45-52.
66. Liu, D., J.T. Perkins, and B. Hennig, *EGCG prevents PCB-126-induced endothelial cell inflammation via epigenetic modifications of NF-kappaB target genes in human endothelial cells.* J Nutr Biochem, 2016. **28**: p. 164-70.
67. Rasheed, Z., N. Rasheed, and H.A. Al-Shobaili, *Epigallocatechin-3-O-gallate up-regulates microRNA-199a-3p expression by down-regulating the expression of cyclooxygenase-2 in stimulated human osteoarthritis chondrocytes.* J Cell Mol Med, 2016. **20**(12): p. 2241-2248.
68. Hussain, T., et al., *Green tea constituent epigallocatechin-3-gallate selectively inhibits COX-2 without affecting COX-1 expression in human prostate carcinoma cells.* Int J Cancer, 2005. **113**(4): p. 660-9.
69. Vankemmelbeke, M.N., et al., *Selective inhibition of ADAMTS-1, -4 and -5 by catechin gallate esters.* Eur J Biochem, 2003. **270**(11): p. 2394-403.

70. Coburn, J.M., et al., *Short-chain fatty acid-modified hexosamine for tissue-engineering osteoarthritic cartilage.* Tissue Eng Part A, 2013. **19**(17-18): p. 2035-44.
71. Almeida, C.R., et al., *Enhanced mesenchymal stromal cell recruitment via natural killer cells by incorporation of inflammatory signals in biomaterials.* J R Soc Interface, 2012. **9**(67): p. 261-71.
72. Mountziaris, P.M. and A.G. Mikos, *Modulation of the inflammatory response for enhanced bone tissue regeneration.* Tissue Eng Part B Rev, 2008. **14**(2): p. 179-86.
73. Brown, B.N., et al., *Macrophage phenotype and remodeling outcomes in response to biologic scaffolds with and without a cellular component.* Biomaterials, 2009. **30**(8): p. 1482-91.
74. Lee, Y., J. Choi, and N.S. Hwang, *Regulation of lubricin for functional cartilage tissue regeneration: a review.* Biomater Res, 2018. **22**: p. 9.
75. Antonas, K.N., J.R. Fraser, and K.D. Muirden, *Distribution of biologically labelled radioactive hyaluronic acid injected into joints.* Ann Rheum Dis, 1973. **32**(2): p. 103-11.
76. Levett, P.A., et al., *Hyaluronic acid enhances the mechanical properties of tissue-engineered cartilage constructs.* PLoS One, 2014. **9**(12): p. e113216.
77. Moulisova, V., et al., *Hybrid Protein-Glycosaminoglycan Hydrogels Promote Chondrogenic Stem Cell Differentiation.* ACS Omega, 2017. **2**(11): p. 7609-7620.
78. Chung, C. and J.A. Burdick, *Influence of three-dimensional hyaluronic acid microenvironments on mesenchymal stem cell chondrogenesis.* Tissue Eng Part A, 2009. **15**(2): p. 243-54.
79. Caron, M.M., et al., *Redifferentiation of dedifferentiated human articular chondrocytes: comparison of 2D and 3D cultures.* Osteoarthritis Cartilage, 2012. **20**(10): p. 1170-8.
80. Brodkin, K.R., A.J. Garcia, and M.E. Levenston, *Chondrocyte phenotypes on different extracellular matrix monolayers.* Biomaterials, 2004. **25**(28): p. 5929-38.
81. Leong, D.J., et al., *Green tea polyphenol treatment is chondroprotective, anti-inflammatory and palliative in a mouse post-traumatic osteoarthritis model.* Arthritis Res Ther, 2014. **16**(6): p. 508.
82. Natarajan, V., B. Madhan, and M.L. Tiku, *Intra-Articular Injections of Polyphenols Protect Articular Cartilage from Inflammation-Induced Degradation: Suggesting a Potential Role in Cartilage Therapeutics.* PLoS One, 2015. **10**(6): p. e0127165.
83. Emoto, S., et al., *Intraperitoneal administration of cisplatin via an in situ cross-linkable hyaluronic acid-based hydrogel for peritoneal dissemination of gastric cancer.* Surg Today, 2014. **44**(5): p. 919-26.

요약 (국문초록)

골 관절염 연골 치료를 위한 염증조절형 히알루론산 하이드로겔

골 관절염(Osteoarthritis)은 한 번 손상을 입게 되면 재생능력이 제한적인 가장 보편적인 근골격계 질환이다. 골 관절염이 발병하게 되면 일련의 면역반응으로부터 만성 염증이 유발되면서 골 관절염이 진행되고 연골이 파괴되게 된다. 이 논문에서는 염증을 조절하고 연골 재생을 촉진하기 위하여 녹차 추출물인 epigallocatechin-3-gallate (EGCG)가 포함된 주사 가능한 하이드로 젤을 사용하였다. EGCG는 염증을 조절하고 radical specie s을 제거할 수 있는 고유한 특성을 가지고 있다. 히알루론산 (Hyaluronic acid)은 연골 세포외기질의 주성분으로써 연골 조직 공학에 일반적으로 사용된다. 본 연구에서는 EGCG를 티라민(tyramine)이 접합된 히알루론산과 젤라틴 겔에 결합시켜 50 μM EGCG 및 5 % w / v 히알루론산으로 구성된 최적 농도의 복합 하이드로 겔을 만들었다. 상기 복합 하이드로 겔은 전염증성 사이토카인인 IL-1β에 대한 연골 세포의 보호 작용을 제공하였다. 또한, 복합 하이드로 겔은 체외에서 연골 재생을 유도하였다. 생체 내 조직 학적 분석은 EGCG-HA/Gelatin 복합 하드로겔이 외과적으로 유도된 골 관절염 동물 모델에서 연골 손실을 최소화한다는 것을 보여주었다. 이 연구는 염증 조절형 히알루론산 기반 하이드로 겔이 골 관절염 치료를 위한 치료 옵션을 제공할 수 있음을 입증한다.

주요어: 히알루론산, 골 관절염, 연골재생, EGCG, 항염증