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Hybrid composites with magnesium-containing glycosaminoglycans as a chondroconducive matrix for osteoarthritic cartilage repair

Guofeng Wu [a,b,c,1], Fenbo Ma [c,1], Zhengwei Liu [a,c], Jiayi Liu [c], Yizhebang Xue [c], Mengdi Zhang [e], Chunyi Wen [f], Bin Tang [c,d,*], Lijun Lin [a,**]

[a] Department of Joint and Orthopedics, Zhujiang Hospital, Southern Medical University, Guangzhou, Guangdong, PR China
[b] Department of Orthopedics, Southern University of Science and Technology Hospital, Shenzhen, Guangdong, PR China
[c] Department of Biomedical Engineering, Southern University of Science and Technology, Shenzhen, Guangdong, PR China
[d] Guangdong Provincial Key Laboratory of Cell Microenvironment and Disease Research, PR China
[e] Department of Sports Medicine, Peking University Shenzhen Hospital, Shenzhen, Guangdong, PR China
[f] Department of Biomedical Engineering, Faculty of Engineering, The Hong Kong Polytechnic University, Kowloon, Hong Kong

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ABSTRACT

The alteration of the extracellular matrix (ECM) homeostasis plays an important role in the development of osteoarthritis (OA). The pathological changes of OA are mainly manifested in the large reduction of components in ECM, like type II collagen and aggrecan, especially hyaluronic acid and chondroitin sulfate and often accompanied by inflammation. Rebuilding ECM and inhibiting inflammation may reverse OA progression. In this work, we developed new magnesium-containing glycosaminoglycans (Mg-GAGs), to create a positive ECM condition for promoting cartilage regeneration and alleviating OA. In vitro results suggested that the introduction of Mg-GAGs contributed to promoting chondrocyte proliferation and facilitated upregulating chondrogenic genes and suppressed inflammation-related factors. Moreover, Mg-GAGs exhibited positive effects on suppressing synovial inflammation, reducing chondrocyte apoptosis and preserving the subchondral bone in the ACLT-induced OA rabbit model. This study provides new insight into ECM-based therapeutic strategy and opens a new avenue for the development of novel OA treatment.

1. Introduction

Osteoarthritis (OA), a degenerative disease caused by multiple factors, is characterized by the gradual degradation of articular cartilage and accompanied by joint pain and dysfunction [1]. OA, as the main cause of severe joint pain and disability in the elderly, is the most common type of joint disease in the world [2]. With the advent of the aging population, more and more people are affected by OA, causing huge economic losses to society [3]. Therefore, practical and effective treatment of OA should be highlighted.

The significant pathological feature of OA is cartilage degeneration and joint inflammation [4]. Among them, the interaction between chondrocytes and the extracellular matrix (ECM) plays an important role in the progression of OA [5]. During the progression of OA, chondrocytes are responsible for the secretion and renewal of the components of ECM under inflammatory conditions [6]. In turn, alterations to the components of the ECM will also affect the metabolic process of chondrocytes [7]. The balance between the synthesis and degradation of matrix components is broken, and the catabolism increases, which ultimately leads to the degradation of the cartilage matrix in the progression of OA [8]. With the progressive degradation of OA cartilage, the type II collagen and aggrecan (ACAN) present in normal joints are gradually lost [9]. It has been reported that glycosaminoglycan (GAG)(Mainly composed of hyaluronic acid (HA) and chondroitin sulfate (CS)) exhaustion is an important factor leading to accelerated cartilage degradation [10]. HA plays an important function in the normal physiological process of joints, not only providing viscosity and elasticity for synovial fluid but also relieving pain [11]. Supplementing exogenous sodium hyaluronate will accelerate the synthesis and secretion of endogenous HA, and enhance the protective

* Correspondence to: B. Tang, Department of Biomedical Engineering, Southern University of Science and Technology, Shenzhen, Guangdong, PR China.
** Corresponding author.
E-mail addresses: tangb@sustc.edu.cn (B. Tang), gost1@smu.edu.cn (L. Lin).
[1] These authors contributed equally to this work.

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effect of synovium and cartilage [12]. In addition, HA can also inhibit the synthesis of cartilage degrading enzymes and promote the production of cartilage matrix [13]. The maintenance of the physiological function of cartilage also depends on the existence of CS [14]. CS can directly act on damaged articular cartilage to achieve the purpose of inhibiting OA and promoting cartilage regeneration [15]. CS can promote OA chondrocytes to produce cartilage proteoglycan involved in chondroprotection thereby alleviating the symptoms of patients with OA [16]. Current studies have found that acellular cartilage matrix, decellularized cell-derived matrix, and inactivated natural cartilage materials have achieved good cartilage repair effects [17,18]. Therefore, we hypothesize that the combined supplement of HA and CS in a certain proportion may be the most optimized way to promote cartilage matrix regeneration.

In addition to the degradation of the cartilage matrix, local inflammation is also a pathological change that needs to be taken into consideration during the progression of OA. Magnesium ions (Mg^{2+}) are widely present in bones and can regulate inflammation [19]. H. Yao et al. found that injection of magnesium chloride into the joint cavity is beneficial to reduce inflammation and inhibit the progression of OA [20]. It has been reported that magnesium exerts an anti-inflammatory effect by enhancing the activity of PI3K/Akt and magnesium supplementation is beneficial to inhibit OA [21,22]. Moreover, it has been found that the increase in magnesium dietary intake was related to better cartilage structure of the knee joint through a cross-sectional study of cartilage structure [23].

Therefore, we propose a new concept to fight OA, which is repairing the deconstructed aggrecans in OA to create a positive tissue regeneration environment for facilitating cartilage tissue growth. In this work, we developed a magnesium-containing HA-CS, a kind of anti-inflammatory complex that can effectively supplement polysaccharides that are lacking in pathological conditions and slow down cartilage degradation by suppressing the local inflammation. We hypothesize that the new functionalized glycosaminoglycans may play a role in promoting chondrogenesis and are expected to be more effective in inhibiting the progression of OA.

2. Methodology

2.1. Materials

Sodium hyaluronate (purity: ≥99 %, Mw: 400–1000 kDa) was purchased from Shanghai Yuanye Biological Technology Co., Ltd. Chondroitin sulfate A (molecular weight 18 kDa) were purchased from Aladdin Industrial Corporation (Shanghai, China). The cation exchange resin used was supplied by Sinopharm Chemical Reagent Co., Ltd. magnesium chloride was purchased from Damao Chemical Reagent Co. Ltd. magnesium hydroxide was supplied by Maclin Chemical Reagent Co., China.

2.2. Preparation of Mg-GAGs

MgCS was fabricated as previously reported [24]. Briefly, the pretreated 732 sodium cation resin was packed in the ion exchange column for absorbing sodium ions in the CS. Then, using $Mg(OH)_2$ to neutralize the resulting liquid to make its pH 6.5. Next, add $MgCl_2$ to the pH-adjusted solution and stir gently for a while to form MgCS. Finally, 95 % ethanol was added into the resulting solution to precipitate MgCS. The precipitation was freeze-dried for at least 24 h.

The literature has reported that the dry weight ratio of extracellular matrix components of cartilage is 15–20 % of collagen, 5–10 % of CS, and 0.05–0.25 % of HA [25]. Therefore, based on the mass ratio of CS and HA in the normal cartilage matrix, we designed Magnesium-containing glycosaminoglycans with different concentration ratios. Mg-GAGs were grouped as follows: high concentration ratio group (MgCS/HA 7.5:1), medium concentration ratio group (MgCS/HA 7.5:0.5), lower concentration ratio group (MgCS/HA 7.5:0.26), low concentration ratio group (MgCS/HA 7.5:0.13). It should be noted that MgCS/HA1, MgCS/HA0.5, MgCS/HA0.26 and MgCS/HA0.13 will be used to refer to the name of the groups in the following description.

2.3. Structural characterization of Mg-GAGs

The structure of magnesium-containing glycosaminoglycans was characterized by Fourier transform infrared (FTIR) (Bruker Vertex 70, Germany) from 4000 cm^{-1} to 500 cm^{-1}.

2.4. Isolation and culture of primary chondrocytes

Human primary chondrocytes were derived from cartilage specimens during total knee arthroplasty in patients with OA with ethical approval issued by the authors' institute. Specifically, wash the cartilage samples thoroughly with sterile phosphate-buffered saline (PBS) at least three times. Then cut the cartilage to the maximum extent with surgical scissors, and digested it with 0.25 % trypsin at 37 °C for 30 min. Subsequently, the digestion was terminated, and the cartilage pieces were washed with Dulbecco's Modified Eagle Medium (DMEM)(HyClone). Next, the cartilage pieces were digested in DMEM containing 0.2 % type II collagenase for 4 h with shaking at 37 °C. In this process, the solution should be pipetted up and down every 30 min to avoid cell and tissue aggregation to ensure adequate digestion. Finally, the cell suspension was filtered into a tube and centrifuged at 1000 rpm for 5 min.

2.5. Cell viability and proliferation tests

The chondrocytes were plated and cultured in DMEM supplemented with 10 % fetal bovine serum (FBS)(Gibco) and 1 % penicillin and streptomycin (PS) in a cell incubator. The chondrocytes used in the subsequent study were expanded through two passages. Cell viability was evaluated with Calcein/PI Live/Dead Viability/Cytotoxicity Assay Kit (Beyotime). Calcein AM stains live cells (green fluorescence) while propidium iodide (PI) stains dead cells (red fluorescence). The cell morphology was observed under a microscope and photographed before Live/Dead staining.

MTT assay was performed to assess the proliferation of chondrocytes cultured in DMEM supplemented with different ratios of Mg-GAGs (MgCS/HA1, MgCS/HA0.5, MgCS/HA0.26, MgCS/HA0.13 and MgCS). Chondrocytes cultured in DMEM were set as the control group. All Mg-GAGs groups were added 144 mg/100 mL of MgCS. The MgCS/HA1, MgCS/HA0.5, MgCS/HA0.26, MgCS/HA0.13 and MgCS groups were supplemented with 19.2 mg/100 mL, 9.6 mg/100 mL, 4.8 mg/100 mL, 2.4 mg/100 mL and 0 of HA respectively. The cells were plated in 96-well plates with 5000 cells per well and six repetitions were performed in each group. To maintain a fixed ratio of Mg-GAGs, the culture medium is not changed throughout the entire cell culture process. DMEM containing 10 % MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (Sigma-Aldrich, USA) was added into each well following incubated for 4 h at 37 °C. After that, the liquid was aspirated and the same volume of DMSO was added into each well in the dark with shaking for 15 min. Finally, the absorbance was detected with a microplate reader (BioRad) at 490 nm.

2.6. Gene expression analysis by qPCR

The effects of different ratios of Mg-GAGs on the gene expression of chondrocytes were evaluated by RT-qPCR. After culturing the cells for 5 days, total RNA was extracted from chondrocytes using RNAiso plus (Takara, Japan) and cDNA was synthesized using a reverse transcription kit (Beyotime) according to the manufacturer's instructions. cDNA was used to amplify the target genes (SOX-9, COLII, ACAN, IL-6, TNF-α, and MMP-9) with β-actin as the reference gene using SYBR Green qPCR Mix (Beyotime) on a Step One Plus real-time PCR System. The $2^{-\Delta\Delta Ct}$ method

was used to calculate the folding change and normalized to the gene expression level of the control group.

2.7. In vivo evaluation of OA treatment in the ACLT-induced OA rabbit model

2.7.1. Surgical procedures

Animal experiments were approved by the Institution Review Board of the Southern University of Science and Technology. Thirty adult male New Zealand rabbits (2.8 ± 0.2 kg, 8 months) were randomly divided into 5 groups. Including normal group, sham group, OA group, ARTZ Dispo group and Mg-GAGs group. A model of OA was created by anterior cruciate ligament transection (ACLT) of the right knee joint. Specifically, the rabbits were anesthetized by isoflurane inhalation. Subsequently, after shaving the right knee joint and using iodophor for disinfection at the operation site, cut the skin along the inside of the patella to open the joint capsule, and transected the anterior cruciate ligament with ophthalmic scissors. A drawer test was then performed to assess the integrity of the ligaments. Fig. S1 (Supplementary Information) displays the surgical procedure. For the sham group, the anterior cruciate ligament was not manipulated or transected. Three days after surgery, each rabbit was given a subcutaneous injection of 100,000 units of penicillin sodium every day to prevent infection. Four weeks after ACLT surgery, weekly intra-articular injections were performed for 5 weeks (The OA group and the sham group were injected with saline (0.1 mL/kg); In the ARTZ Dispo group, commercial sodium hyaluronate was injected; The Mg-GAGs group was injected with Mg-GAGs (0.3 mL of Mg-GAGs was injected into the joint cavity, in which the concentration of MgCS was 288 mg/100 mL and HA was 4.8 mg/100 mL). No treatment was performed in the normal group). Finally, the rabbits were sacrificed to obtain knee cartilage and synovial tissue by overdose anesthesia with isoflurane and intravenous injection of 10 % KCl. OARIS score was performed on the collected femoral condyle and tibial plateau cartilage according to the previous literature [26].

2.7.2. Microcomputed tomography (micro-CT)

The changes in articular subchondral bone before and after treatment were evaluated by micro-CT analysis. The specimens were fixed with 4 % paraformaldehyde for 48 h. The scan was then performed on a high-resolution micro-CT imaging system. The same parameters were used for each sample (scanning thickness: 18 μm, scanning voltage: 80 kV, current: 100 μA). A phantom of fixed densities (250 mg/cm^3 and 750 mg/cm^3) was used for calculating the bone mineral density (BMD) of subchondral cancellous bone. The bone mass density (BMD), trabecular number (Tb. N), trabecular thickness (Tb. Th) and trabecular separation (Tb. Sp) of subchondral bone were quantitatively analyzed.

2.7.3. Histological evaluation

The cartilage tissue specimens were fixed with 4 % paraformaldehyde for 48 h and decalcified with 10 % EDTA solution for one month with changing the decalcification solution once a week. After decalcification, routine dehydration and paraffin embedding were performed to make 5 μm-thick serial sections, which were histologically stained, including HE staining and Safranin O fast green staining. Similarly, the synovial tissue was fixed, dehydrated, and embedded in paraffin to make continuous paraffin sections with a thickness of 5 μm, which were performed HE staining. All the stained sections were observed under a microscope and histologically scored. The scoring standards refer to the methods in the literature [26].

2.7.4. Hoechst staining

The cartilage paraffin sections were dewaxed and hydrated. The Hoechst kit was used to detect the apoptosis of chondrocytes. Briefly, the sections are deparaffinized and hydrated and stained for 5 min with 0.5 mL Hoechst 33258 staining solution. Subsequently, remove the staining solution and wash twice with PBS for 3 min each time. Finally, a blue nucleus can be detected under a fluorescence microscope. The excitation wavelength was 350 nm and the emission wavelength was 460 nm.

2.8. Statistical analysis

Statistical analysis was performed using SPSS19.0 software, and the data were expressed as mean ± standard deviation (SD). The comparison among multiple groups was used a one-way analysis of variance (ANOVA). Values shown are mean ± SD for triplicate cultures $^*p < 0.05$ or $^{**}p < 0.01$ or $^{***}p < 0.001$ stands for significant difference.

3. Results and discussions

3.1. The structural characterization of Mg-GAGs

The FTIR spectra of HA, MgCS and Mg-GAGs were displayed in Fig. 1. The peaks at 3300–3500 cm^{-1} in the FTIR spectrograph, corresponding to the NH and OH stretching vibrations. While these peaks were shifted in Mg-GAGs, which indicated that the preparation of Mg-GAGs was not a simple physical mixing and there may exist intermolecular hydrogen bonding. It has been reported that the addition of chondroitin sulfate into hyaluronan may create more intermolecular hydrogen bonds thereby contributing to increasing the stability of the entire complex network [27].

3.2. Effect of Mg-GAGs on cell morphology, cytotoxicity and proliferation

The morphology of chondrocytes exerts a great influence on chondrocyte phenotype stability and it plays an important role in OA pathology and cartilage repair [28]. Fig. 2A displays the morphology of chondrocytes cultured in DMEM supplemented with MgCS and different ratios of Mg-GAGs (MgCS/HA1, MgCS/HA0.5, MgCS/HA0.26 and MgCS/HA0.13). As shown in this figure, the ratio of Mg-GAGs does not affect the morphology of the cells. This should be due to the synergistic effect of CS and HA plays a positive role in maintaining cell morphology. Previously reported that the presence of chondroitin-6-sulfate facilitated the maintenance of the chondrocytic phenotype throughout the culture period [29]. Moreover, a low concentration of hyaluronic acid could stabilize the chondrocytic phenotype [30]. Thus, Mg-GAGs could maintain the phenotypic stability of chondrocytes. Cytotoxicity Assay was used to evaluate the effects of Mg-GAGs on the chondrocytes' viability for 3 days (Fig. 2B). It suggested that cell viability in MgCS/HA1, MgCS/HA0.5 and MgCS/HA0.26 groups were improved when compared with the other groups. This means that Mg-GAGs could

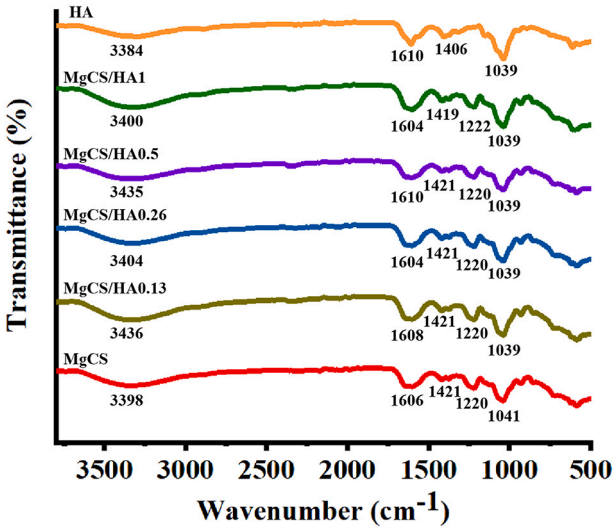

Fig. 1. FTIR spectra of HA, MgCS and Mg-GAGs.

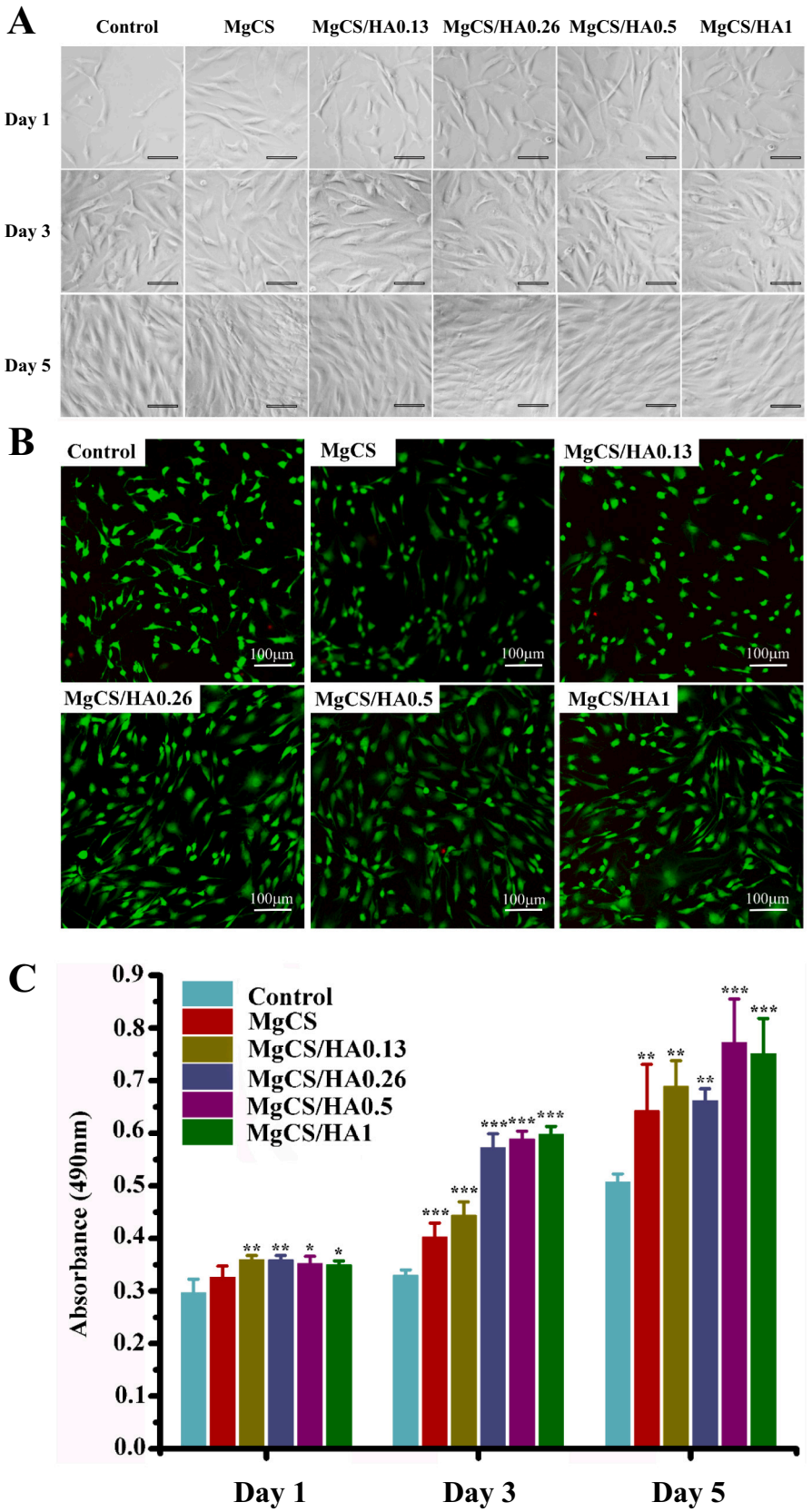

Fig. 2. Effect of Mg-GAGs on morphology (A), cytotoxicity (B) and proliferation (C) to chondrocytes. Scale bar: 100 μm. *$p < 0.05$ or **$p < 0.01$ or ***$p < 0.001$ stands for significant difference when compared with the control group.

increase chondrocyte viability in an HA-concentration-dependent manner. Glycosaminoglycan concentration was considered to significantly affect cell behavior [31]. Moreover, chondrocytes in all Mg-GAGs groups proliferated over time; whereas the higher concentration of HA in Mg-GAGs has a more significant effect on stimulating cell proliferation. It has been reported that supplementation with CS and/or HA has positive effects on chondrocyte proliferation and a dose-dependent relationship with certain amounts of added HA stimulating chondrocyte proliferation [32,33]. Thus, the cell proliferation results suggested that Mg-GAGs with an optimal HA concentration can effectively improve the proliferation of OA chondrocytes.

3.3. In vitro gene expression analysis

Using qPCR, we initially evaluated the mRNA expression of chondrogenic genes (ACAN, collagen II and SOX-9) in different groups. The deposition of aggrecan (a component of the extracellular matrix of cartilage) encoded by ACAN is considered to be the hallmark of chondrogenesis [34]. Chondrocytes in Mg-GAGs0.13 treated cultures displayed a >2-fold increase of ACAN, indicating Mg-GAGs0.13 could exert a more beneficial effect on chondrogenesis (Fig. 3). This may be due to the more obvious stimulating effect of Mg-GAGs with this concentration of HA on chondrocytes. Previously reported that the amount of glycosaminoglycan seemed to be dependent on HA content and low HA supplementation leads to higher glycosaminoglycan deposition during chondrogenesis [35]. SOX-9 is coexpressed with collagen type II during chondrogenesis and it accompanies the differentiation of chondrocytes [36]. Collagen II and SOX-9 were expressed in all groups, with the highest level being in the MgCS/HA0.13 group (Fig. 3). Chondrocytes in Mg-GAGs0.13 treated cultures displayed a >2-fold increase of collagen II and chondrocytes showed a >6-fold up-regulation of SOX-9 gene expression. As reported in the previous study, lower HA could lead to a higher stimulation of chondrogenesis [37]. Additionally, magnesium was also reported to be beneficial in promoting cartilage matrix synthesis [38]. Thus, Mg-GAGs0.13 with lower HA showed upregulated expression levels of chondrogenesis-related genes.

We also examined the expression levels of inflammation-related genes (IL-6, TNF-α, and MMP-9). The pro-inflammatory cytokines, IL-6 and TNF-α, are considered to be the most important components to maintaining the inflammatory response in OA and they also play a role in the up-regulation of MMP gene expression. The significant increase of IL-6 and TNF-α levels in OA produces significant MMP-9, which leads to the degradation of articular cartilage extracellular matrix proteins [39]. Chondrocytes cultured in the presence of MgCS/HA0.13 demonstrated a decrease in inflammation-related genes (IL-6, TNF-α, and MMP-9) expression as compared to the control cultures and other Mg-GAGs treated cultures thus demonstrating MgCS/HA0.13 should be more effective in promoting aggrecan and collagen formation and suppressing chondrocyte inflammation. Magnesium is known to reduce inflammation in the knee synovium [40]. In addition, the anti-inflammatory and chondroprotective effects of HA suggested its potential role in attenuating joint damage [41]. Previously reported that HA could inhibit inflammatory reactions in a dose-related fashion [42]. Therefore, relative inflammatory cytokines expression levels of chondrocytes in different ratios of Mg-GAGs demonstrated varying levels. In summary, the MgCS/HA0.13 group showed more excellent chondrogenesis-promoting and inflammation-suppressing capability, therefore MgCS/HA0.13 was applied in the subsequent in vivo studies.

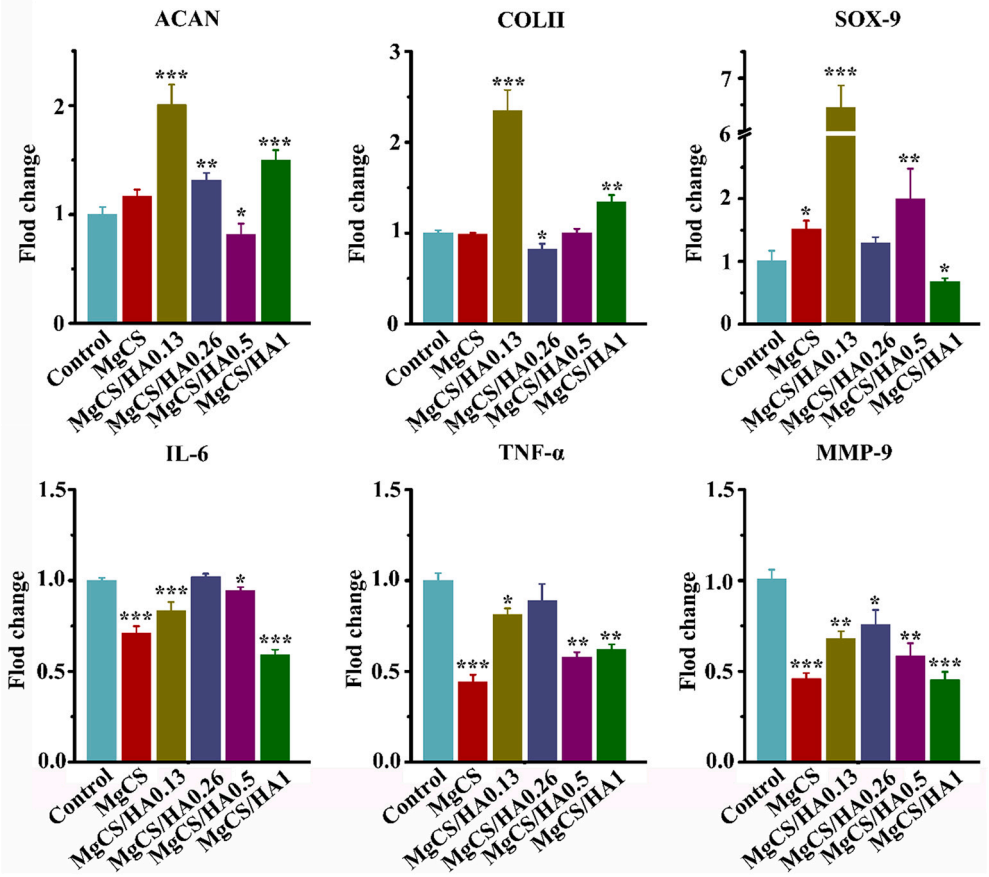

Fig. 3. The relative gene expression levels of chondrocytes after culture in DMEM supplemented with MgCS and different ratios of Mg-GAGs. *$p < 0.05$ or **$p < 0.01$ or ***$p < 0.001$ indicates a significant difference relative to the control group. All data represent mean ± S.D.

3.4. Mg-GAGs accelerates cartilage regeneration in rabbits ACLT model

A preliminary animal study using rabbits' OA model was performed to assess the effects of Mg-GAGs on accelerating cartilage regeneration. Macroscopic analysis of the tibial plateau and femoral condyle in the rabbits demonstrated evidence of the remaining damage in OA animals while rabbits treated with ARTZ Dispo or Mg-GAGs displayed smooth cartilage healing (Fig. 4A). Intact cartilage of knee joints with smooth and bright surfaces was observed in the normal and sham groups. However, in the OA group, the femoral condyle displayed a softened and rough surface with injuries. The conditions of cartilage in the ARTZ Dispo or Mg-GAGs groups were better than those in the OA group. The gross morphological scoring of articular cartilage in different groups was adopted to evaluate cartilage damage and the results were shown in Fig. 4D. The higher the score, the higher the severity of OA. With the establishment of the rabbits' OA model, the scores were all increased. In the ARTZ Dispo and Mg-GAGs groups, the scores were lower than those in the OA group. The result indicated that both ARTZ Dispo and Mg-GAGs could promote OA healing whereas Mg-GAGs seemed more effective in repairing articular cartilage. Moreover, the quality of the repair was analyzed histologically with HE (Fig. 4B) and safranin-O/fast green (Fig. 4C) staining. To clarify the histopathological changes in different groups, we quantitatively evaluated the changes by the OARSI scoring system. The higher the values, the more advanced cartilage degeneration (Fig. 4E).

Researchers have discovered that subchondral bone plays an important role in OA [43]. It has many blood vessels to provide nutrition and oxygen to the articular cartilage and take away waste products. Therefore, the health and function of the subchondral bone will affect the health of the cartilage above it. Following the positive results observed in the macroscopic and histological analysis, it was hypothesized that treatment of OA with Mg-GAGs can improve the quality of the

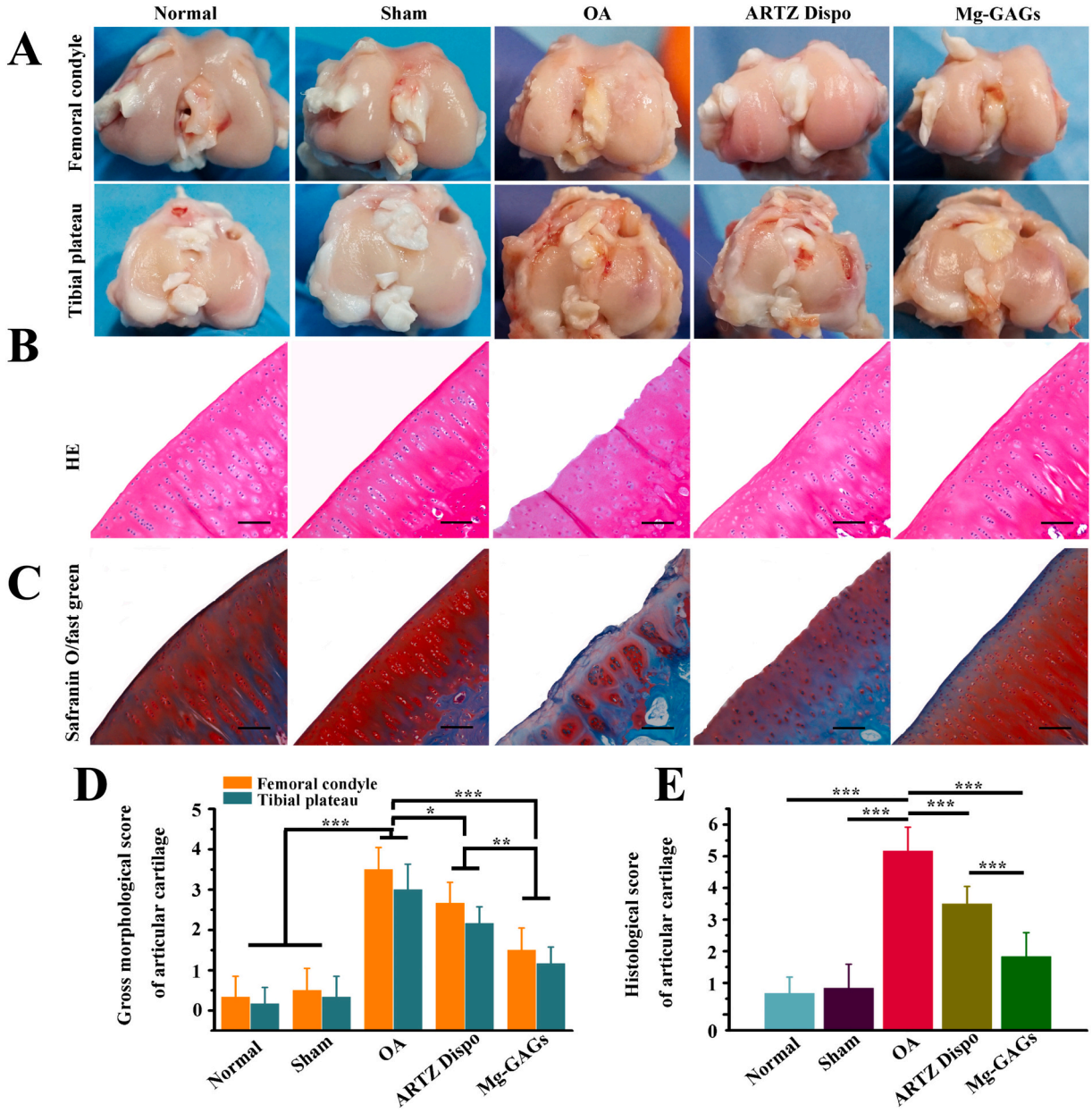

Fig. 4. Effect of Mg-GAGs on cartilage regeneration. A: Macroscopic images of the femoral condyle and tibial plateau; B: HE staining; C: Safranin O fast green staining; D: The gross morphological score of articular cartilage; E: The histological score. Scale bar: 100 μm. $^{*}p < 0.05$ or $^{**}p < 0.01$ or $^{***}p < 0.001$ indicates a significant difference. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

subchondral bone.

Fig. 5A exhibits the representative micro-CT images of the trabecula of subchondral bone in different groups. As shown in the figure, the bone trabecula in the normal and sham groups was arranged neatly with no obvious cavity and bone collapse. Meanwhile, the bone trabecula in the ARTZ Dispo group was arranged slightly neatly, and the bone trabeculae did not collapse. However, in the OA group, the bone trabecula was hollow, sparse, and irregularly arranged. This indicated that ACLT surgery has extensively deteriorated the articular cartilage. Moreover, the subchondral bone beneath the damaged cartilage was thickening, loss of cancellous bone and the formation of osteophyte can be seen in the OA group.

The quantitative determination of subchondral bone healing (bone mass density (BMD), trabecular number (Tb. N), trabecular thickness (Tb. Th) and trabecular separation (Tb. Sp) of subchondral bone) were shown in Fig. 5B–E. The BMD value in the Mg-GAGs-treated group was significantly increased relative to both ARTZ Dispo and OA groups. No significant differences in BMD were found between normal and sham knees. The BMD in normal and sham knees was greater than the BMD in knees after surgery.

Tb.N and Tb. Th assessments of the subchondral bone appeared comparatively higher for Mg-GAGs-treated OA rabbits relative to the ARTZ Dispo and OA groups. Moreover, a significant decrease in the Tb. Sp of the subchondral bone of Mg-GAGs group (approximately 500 μm) compared to ARTZ Dispo and OA groups (>600 μm) was observed. This suggested that there were thicker trabecular bone deposits in the Mg-GAGs group with lower marrow space thicknesses between the trabeculae. These collective data indicated that Mg-GAGs can effectively preserve the subchondral bone thereby promoting cartilage regeneration. Overall, micro-CT analysis demonstrated that the Mg-GAGs exhibited a stimulating effect on subchondral bone healing.

Fig. 6A shows HE staining of synovial tissue. There was no obvious inflammation and synovial hyperplasia in the synovial tissues in knee joints of rabbits in the normal and sham groups. However, in the OA group, obvious synovial hyperplasia or fibrosis was observed, which suggested OA models were successfully established. Meanwhile, inflammatory infiltration and thickened blood vessel wall were also found in the OA group. Compared to the OA group, the rabbits in the ARTZ Dispo group displayed reduced synovial tissue hyperplasia and inflammatory infiltration, though thickened synovium still existed. Interestingly, the Mg-GAGs group showed notably decreased synovial tissue hyperplasia and inflammatory infiltration, without thickened synovium

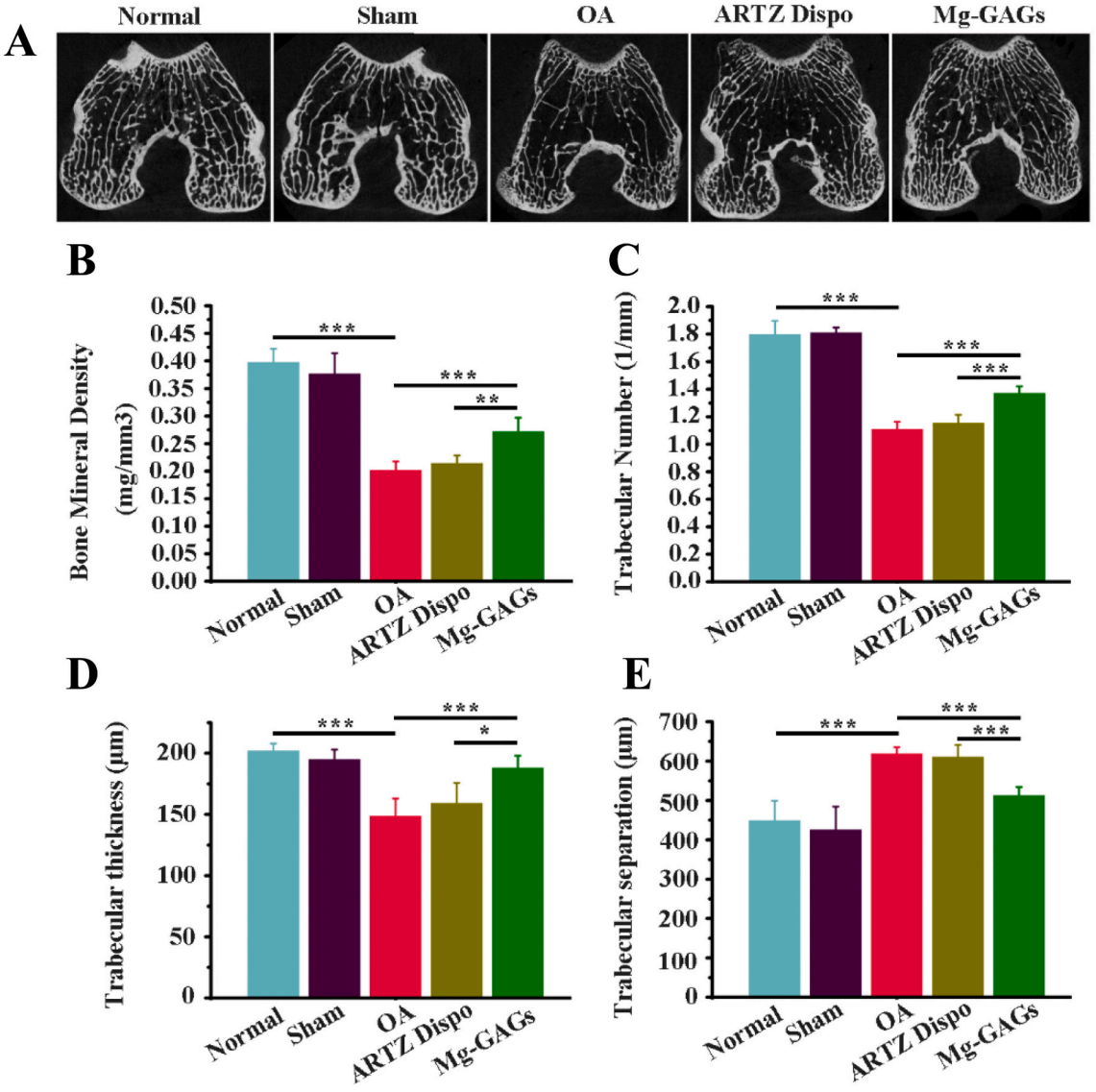

Fig. 5. A: Representative micro-CT images within the subchondral bone; B–E: Quantitative parameters including bone mass density (BMD), trabecular number (Tb. N), trabecular thickness and trabecular separation of subchondral bone. (*$p < 0.05$ or **$p < 0.01$ or ***$p < 0.001$ indicates a significant difference).

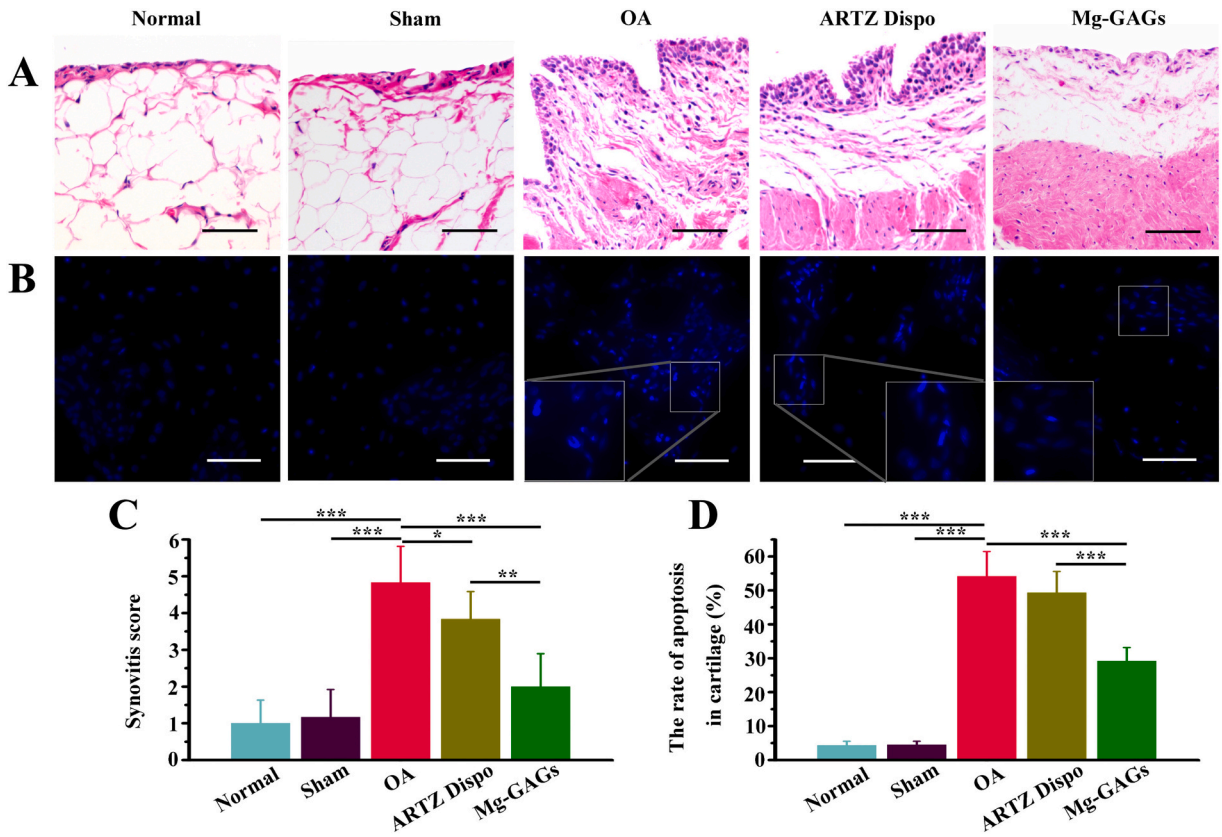

Fig. 6. A: HE staining of synovial tissue. Scale bar: 75 μm; B: Hoechst staining was used to observe apoptosis conditions of chondrocytes; C: Inflammation scores of synovitis; D: Quantitative analysis for the apoptosis of chondrocytes. Scale bar: 50 μm. *$p < 0.05$ or **$p < 0.01$ or ***$p < 0.001$ indicates a significant difference.

in knee joints, when compared with both in the OA and the ARTZ Dispo groups.

The degenerative and inflammatory processes can be reflected by changes in the synovial membrane. The severity of inflammation in the synovial tissue was scored and the results were summarized in Fig. 6C. Severe synovitis in the synovial tissue of knee joints was identified in the OA group, with inflammation scores higher than the other groups. The rabbits in the ARTZ Dispo group showed medium synovitis, the inflammation scores of which were higher than those in the normal, sham and Mg-GAGs groups. The rats in the Mg-GAGs group displayed mild synovitis in the synovial tissue of knee joints, indicating that Mg-GAGs could relieve the inflammation of the synovium.

Chondrocyte apoptosis is one characteristic of osteoarthritic cartilage, which may contribute to the structural and metabolic changes found in osteoarthritic cartilage. To evaluate the effects of Mg-GAGs on the inhibition of chondrocyte apoptosis, hoechst staining was performed to observe the apoptosis conditions of chondrocytes (Fig. 6B). A few apoptotic chondrocytes were found in the normal and sham groups while plenty of apoptotic chondrocytes existed in the OA group. Compared with the normal and sham groups, the apoptosis rate in the OA and the ARTZ Dispo groups was higher (Fig. 6D). When compared with the OA and the ARTZ Dispo groups, the apoptosis rate of chondrocytes was reduced in the Mg-GAGs group. These results suggested that Mg-GAGs could suppress the apoptosis of chondrocytes in knee joints of rabbits with OA.

In vivo study, we tried to identify an alternative and complementary therapeutic strategy for OA healing using the ACLT-induced OA rabbit model. The results suggested that the cartilaginous tissue repair capability in Mg-GAGs group was better overall than in ARTZ Dispo and OA groups. It should be admitted that due to the limit of the scope of this work, we can not figure out all the possible mechanisms for Mg-GAGs on OA. However, the lack of HA and CS in OA progression has been well-documented, and HA and CS both have a positive role in maintaining cartilage, e.g. HA has a beneficial effect on lubrication, anti-inflammatory and chondroprotective effects [44] and CS elicited an anti-inflammatory effect at the chondral and synovial levels and promoted chondrogenesis [45,46]. The Design logic of Mg-GAGs is simple, to make up the HA and CS that be lost due to the OA progression, so that creates a healthy ECM condition before tissue regeneration by introducing CS and HA with optimal ratio. The significant improvement of the entire joint after Mg-GAGs treatment proves the success of Mg-GAGs design, and the in vitro study shown previously provides us certain hints for how the Mg-GAGs exert positive effects during the OA, such as anti-inflammation, anti-apoptosis, etc. However, we do believe Mg-GAGs should have more positive effects than we reported in the present study, and are worth being further investigated in the future.

Taken together, the above findings support the hypothesis that the developed Mg-GAGs promote improvement in cartilaginous repair. Therefore, the developed Mg-GAGs may function as a potential chondroconducive matrix that supports cartilage regeneration for bringing broad prospects in clinical applications and the concept that developed functional GAGs for OA treatment or osteoarthritic cartilage repair should be highlighted in tissue engineering.

4. Conclusion

The results from this work demonstrated that developed Mg-GAGs can provide a pro-regenerative environment to support the regeneration of articular cartilage and subchondral bone following ACLT-induced OA rabbits. The Mg-GAGs stimulated chondrocytes to produce aggrecan, collagen type 2 and SOX-9, downregulating the cells to produce inflammatory factors and resulting in cartilage generation. Collectively, we believe that the developed Mg-GAGs is expected to serve as a direct treatment toward accelerating OA healing and

improving the quality of cartilage repair. Our strategy to improve the efficiency of OA treatment is to use a combination of natural, HA- and CS-based polymers to enhance regeneration of the cartilage extracellular matrix and inhibit inflammation and chondrocyte apoptosis. This work may provide a concept for designing practical biomaterials targeting rebuilding ECM and improving subchondral bone quality thereby promoting OA healing.

Abbreviations

ECM	extracellular matrix
OA	osteoarthritis
ACAN	aggrecan
HA	hyaluronic acid
CS	chondroitin sulfate
GAG	glycosaminoglycan
Mg-GAGs	magnesium-containing glycosaminoglycans
DMEM	Dulbecco's Modified Eagle Medium
PBS	phosphate-buffered saline
FBS	fetal bovine serum
PS	penicillin and streptomycin
ACLT	anterior cruciate ligament transection
BMD	bone mass density
Tb. N	trabecular number
Tb. Th	trabecular thickness
Tb. Sp	trabecular separation

CRediT authorship contribution statement

Guofeng Wu: Conceptualization, Methodology, Investigation, Validation, Formal analysis, Writing original draft; Fenbo Ma: Conceptualization, Methodology, Investigation, Validation, Formal analysis, Writing-Review & Editing; Zhengwei Liu:Methodology, Investigation, Formal analysis; Yizhebang Xue: Investigation; Mengdi Zhang: Investigation; Chunyi Wen: Investigation; Bin Tang: Conceptualization, Writing-Review & Editing, Funding acquisition, Project administration, Supervision; Lijun Lin: Conceptualization, Project administration, Resources, Supervision.

Declaration of competing interest

There are no conflicts to declare.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijbiomac.2022.08.071.

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