Bio-Design and Manufacturing (2021) 4:33–43
https://doi.org/10.1007/s42242-020-00090-8

RESEARCH ARTICLE

Effects of bionic mechanical stimulation on the properties of engineered cartilage tissue

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Received: 22 June 2020 / Accepted: 29 July 2020 / Published online: 19 October 2020

Abstract

Tissue-engineered cartilage (TEC) remains a potential alternative for the repair of articular cartilage defects. However, there has been a significant different between the properties of TEC and those of natural cartilage. Studies have shown that mechanical stimulation such as compressive load can help regulate matrix remodelling in TEC, thus affecting its biomechanical properties. However, the influences of shear induced from the tissue fluid phase have not been well studied and may play an important role in tissue regeneration especially when integrated with the compressive load. Therefore, the aim of this study was to quantitatively investigate the effects of combined loading mechanisms on TEC in vitro. A bespoke biosimulator was built to incorporate the coupled motion of compression, friction and shear. The specimens, encapsulating freshly isolated rabbit chondrocytes in a hydrogel, were cultured within the biosimulator under various mechanical stimulations for 4 weeks, and the tissue activity, matrix contents and the mechanical properties were examined. Study groups were categorized according to different mechanical stimulation combinations, including strain (5–20% at 5% intervals) and frequency (0.25 Hz, 0.5 Hz, 1 Hz), and the effects on tissue behaviour were investigated. During the dynamic culture process, a combined load was applied to simulate the combined effects of compression, friction and shear on articular cartilage during human movement. The results indicated that a larger strain and higher frequency were more favourable for the specimen in terms of the cell proliferation and extracellular matrix synthesis. Moreover, the combined mechanical stimulation was more beneficial to matrix remodelling than the single loading motion. However, the contribution of the combined mechanical stimulation to the engineered cartilaginous tissue matrix was not sufficient to impede biodegradation of the tissue with culture time.

Keywords Bionic mechanical stimulation · Tissue-engineered cartilage · Biosimulator · Shear

Introduction

Bone and rheumatoid arthritis are common diseases that affect the articular cartilage tissue of adults due to arthritis, trauma or tumours. Because articular cartilage has no blood vessels, lymphatic vessels or nerves, chondrocytes have poor proliferation rate and a low regeneration capacity and thus unable to migrate to damaged sites, which makes it difficult to repair defects on its own [1, 2]. Clinical repair methods include cartilage transplantation [3], subchondral bone drilling and microfracture. Cartilage transplantation involves preparation and preservation of a graft, coupled with maintenance of the cells during the operation [3]. Although subchondral bone drilling and microfracture [4] can achieve a short-term treatment effect, the regenerated cartilage is fibrocartilage which has an inferior property than hyaline cartilage and cannot hold long in the joint, which might result in further joint degeneration in the long run.

Tissue engineering has provided a new treatment method for osteochondral repair [5]. Generally, an engineered tissue is composed of a scaffold, cells and necessary growth factors [6, 7], which can effectively repair cartilage damage and restore cartilage function [8]. At present, although

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tissue engineering can produce hyaline cartilage similar in appearance/morphology to articular cartilage, its properties, such as compression modulus and permeability coefficient, are still far from those of natural cartilage [9–11]. Normal articular cartilage is always subjected to complex mechanical loads during daily activities [12]. The poor environment of the joint site after scaffold implantation can easily lead to hardening and cracking of the host, and the tissue itself degenerates due to the poor environment and nutrient deficiency [13]. Therefore, researchers have attempted to improve the performance of tissue-engineered cartilage via external action. Studies have found that proper stimulation can affect the cartilaginous tissue morphology and gene expression; regulate the synthesis and secretion of matrix components such as GAGs and collagen [14–17]; and play an important role in maintaining the normal structure and function of articular cartilage. To date, most studies of the effects of mechanical stimuli on tissue cartilage have focused on a single type, such as fluid shear stress, liquid pressure, stretching and simple compression [18–23]. Omata et al. [19] demonstrated that cyclic compressive loading can benefit tissue engineering chondrocyte matrix remodelling and its mechanical properties. Tony et al. applied fluid shear to an agarose scaffold compounded with porcine articular chondrocytes and found that the fluid shear force helped promote the secretion of tissue-engineered cartilage matrix and improved the mechanical properties [22]. In general, articular motion is the combination of compression load and shear stress [24]. Therefore, it is of great significance to study the effects of composite forms of mechanical stimulation on tissue-engineered cartilage, to find an optimized stimulus to regulate and improve the structure and mechanics of tissue during in vitro culture [25].

In this paper, tissue-engineered cartilage samples were constructed through encapsulation of freshly derived chondrocytes, and various loading schemes were applied to the engineered cartilaginous tissue via a bespoke biosimulator to study the effects of combined mechanical stimulation on tissue morphology and properties during long-term in vitro culture.

Equipment and materials

Bespoke biosimulator

A biosimulator was designed and built in-house for functional stimulation of engineered cartilaginous tissue and is shown in Fig. 1. The simulator includes three parts: a mechanical stimulation actuator, a controller part and a sterile environment. The actuator is used for clamping the sample as well as directly applying stimulation to the sample; the controller is used for inputting the motion and loading parameters to control the movement of the actuator;

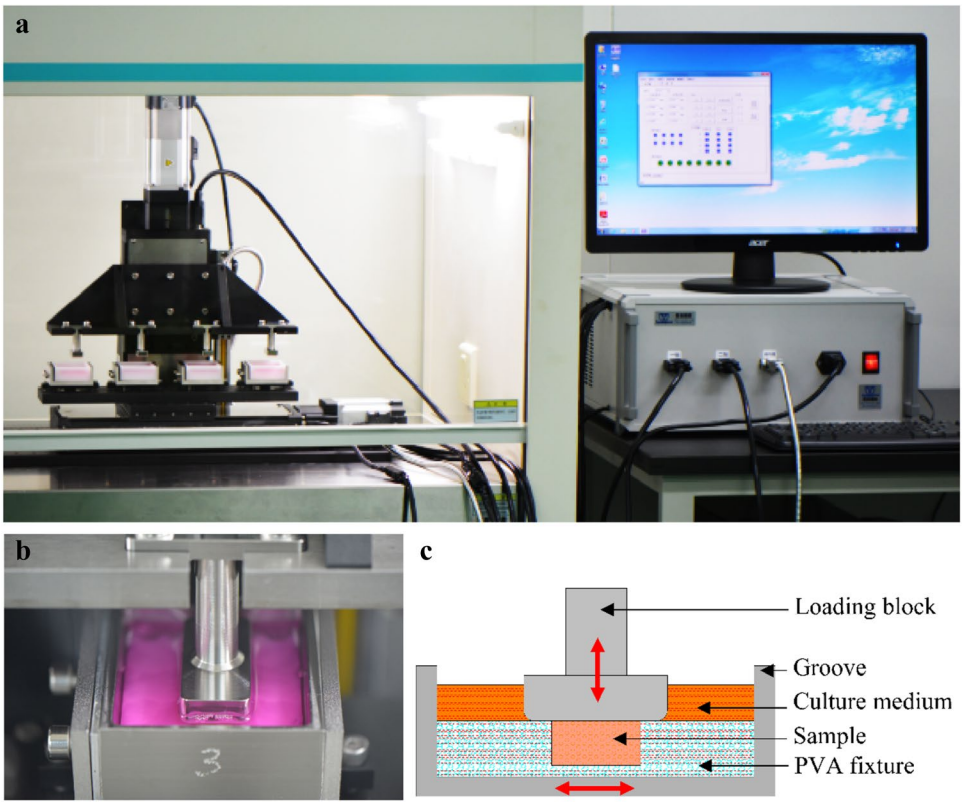

Fig. 1 Tissue-engineered cartilage biosimulator (**a** Bespoke tissue-engineered cartilage biosimulator; **b** station and fixture of the biosimulator; **c** schematic drawing of the loading scheme)

the sterile environment ensures the absence of contamination throughout the dynamic culture process. The mechanical stimulation actuator is mainly composed of horizontal motion unit and vertical loading unit. The vertical loading unit can apply dynamic compressive load to the tissue samples by reciprocating the indenter vertically at a specific frequency and amplitude relative to the experimental samples. The lower platform holding four identical stations of PVA fixtures for the tissue samples can be driven to reciprocate horizontally, which can generate shear to the interfacing surface of the tissue samples and the indenter tissue. The vertical stroke was 35 mm, the horizontal stroke was 28 mm, the maximum speed of vertical and horizontal motion was 20 mm/s, and the vertical loading frequency was 2 Hz.

Cell isolation and encapsulation

Cell isolation

Chondrocytes derived from the limbs of one-day-old New Zealand white rabbits were selected in this study. The rabbits were sacrificed by cervical dislocation, and the cartilage was dissected from the elbow joints of the forelegs and knee joints of the hind limbs which were disinfected with 75% alcohol. Samples were placed in a petri dish with phosphate buffer saline (PBS) and cut into pieces. After being washed 3 times with PBS, the specimens were digested at 37 °C, in Dulbecco's modified eagle medium (DMEM) solution containing 10 mM Hepes–NaOH, pH 7.4, 2% fetal calf serum (Seromed, Berlin) 2 mM glutamine, 500 U ml^{-1} penicillin, 500 U ml^{-1} streptomycin and 125 U ml^{-1} collagenase (Sigma C6885) for 4 h under constant oscillation. The supernatant in the centrifuge tube was removed at 4-h intervals, and the suspension was filtered off with a cell sieve (FALCON, 352340). The supernatant was transferred into a 15 ml centrifuge tube, centrifuged at 1300 rmp for 5 min and then precipitated and prepared for encapsulation [26, 27].

Cell encapsulation

The preparation process employed a casting methodology and is shown in Fig. 2. Sodium alginate solution [2% (m/v), sodium alginate from brown algae, st2116-100 g, Reagent Grade, Life Science Products and Services] was used to prepare the sodium alginate cell suspension with a chondrocyte density of 3×10^6 cells/ml. Chondrocytes and sodium alginate solution were added to the 50 ml centrifuge tube according to the cell density(3×10^6 cells/ml), and the centrifuge tube was placed on a vortex mixer to oscillate at high speed to homogenize the suspension. The mould was

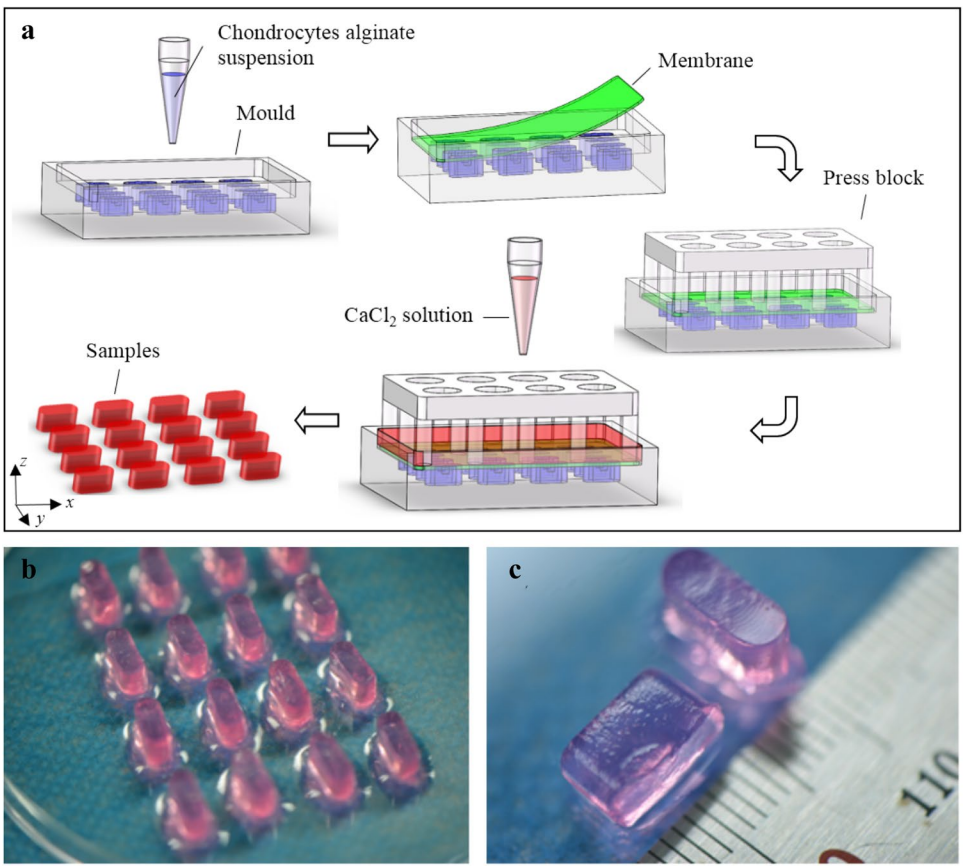

Fig. 2 Preparation of tissue-engineered cartilage (**a** Preparation of tissue-engineered cartilage; **b/c** samples of the tissue-engineered cartilage)

removed from the disinfectant, washed with normal saline 3 times and placed in an ultraclean table for air drying. Afterwards, the sodium alginate cell suspension was injected into the mould along the sidewall of the mould cavity. A sterile membrane was laid on the top surface of the mould to eliminate air bubbles. Next, a pressure plate was placed on the filter membrane and pressed on the mould parting surface. $CaCl_2$ solution (Tianjin Hengxing Chemical Reagent Manufacturing Co., Ltd.) was injected into the mould through the liquid filling hole on the pressure plate. The volume ratio of the calcium alginate cell suspension to the CaCl2 solution was 1: 1. The mould was transferred into the cell incubator and incubated for 4 h. Then, the press plate was removed, the $CaCl_2$ solution was suctioned out, the filter membrane was gently opened, and tissue-engineered cartilage of 9 mm × 4 mm × 5 mm (outside dimensions) was obtained by stripping.

Bionic mechanical stimulation

In human daily activities, the mechanical stimulation of articular cartilage primarily involves pressure, friction and shear. The main forms of articular cartilage stress vary under different motion states. To identify the roles played by the compressive load, frictional velocity and loading frequency, seven combined loading strategies were planned, as shown in Table 1.

Among them, in group P, a simple compressive load was applied during the dynamic culture process to simulate the pressure of the articular cartilage during standing. During the dynamic culture process, a sliding compressive load which along the X-axis of the samples was applied in the S, SA2, SA1, SF2 and SF1 groups to simulate the combined effects of compression, friction and shear on articular cartilage during human movement, and a controlled variable method was adopted in which the compression amplitude changes in S, SA2 and SA1 were 20%, 10% and 5%, respectively. The frequency changes in the S, SF2 and SF1 groups were 1 Hz, 0.5 Hz and 0.25 Hz. The loading block was set to travel for the same distance in different loading groups; thus, the friction velocity was increased along with the loading frequency. The experimental groups were dynamically cultured in the biosimulator for 1.5 h per day. In addition to the exercise time, the samples were placed in a flat culture flask in a CO_2 incubator for static culture. For comparison, the control group C was only subjected to static culture in the incubator throughout the experiment. The samples were cultured for up to 4 weeks and were examined at the midpoint.

Table 1 Experimental groupings and loading parameter settings for biomimetic mechanical stimulation

Groups	Compressive strain amplitude	Frictional velocity (mm s^{-1})	Loading frequency (Hz)
P	20%	0	1
S	20%	10	1
SF2	20%	5	0.5
SF1	20%	2.5	0.25
SA2	10%	10	1
SA1	5%	10	1
C	0	0	0

Tissue examination methodologies

Tissue activity test

The freezing slice method was used to obtain 200 μm thickness slices. A LIVE/DEAD Viability/Cytotoxicity Kit (L3224, Thermo, USA) was utilized to study the cell viability of the engineered cartilaginous tissue. The living cells were stained with a green fluorescent marker, and the dead cells were stained with a red marker. The engineered cartilaginous tissue was collected, fixed in 10% neutral buffered formalin, processed using standard histological techniques and stained with haematoxylin and eosin (H&E). Laser scanning confocal microscopy (LSCM) (A1, Nikon, Japan) was used to image the stained cells.

Cell proliferation

Cell proliferation was determined with Cell Counting Kit-8 (CCK-8) according to the manufacturer's protocol (Dongren Chemical Technology Shanghai Co., Ltd). CCK-8 allows convenient assays using WST-8 reagent, which is bioreduced by cellular dehydrogenases to an orange formazan product. The amount of formazan produced is directly proportional to the number of living cells [28]. CCK-8 solutions were added to each sample, and the incubation was continued for an additional 4 h. The experiment was performed with five wells per group. The absorbance of each well was measured using a microplate reader (1510, Thermo, USA).

Remodelling of ECM

The secretion of biomolecules from extracellular matrix in tissue-engineered cartilage was evaluated by detecting the collagen and glycosaminoglycan (GAG) content in the matrix. The samples were dissolved in sodium citrate solution (55 mM/l sodium citrate (analytical grade, Tianjin Tianli Chemical Reagent Co., Ltd.) + 0.15 M/l sodium chloride solution (analytical grade, Hongyan Reagent Factory, Tianjin Hedong District)) with ultrapure water at 4 °C. The supernatant obtained by centrifugation was a solution of GAGs and hydroxyproline in the further matrix. The sediment was dissolved after centrifugation in papain solution

(50 μg/ml papain (P0150, Xi'an Guoan Biotechnology Co., Ltd.)) prepared with PBS + 5 m M/l EDTA (analytical grade, Tianjin Shengao Chemical Reagent Co., Ltd.) + 5 mM/l L-cysteine (C108237-100 g, Aladdin) with PBS at 60 °C. Then, the supernatant obtained by centrifugation was a solution of cell-associated matrix GAGs and hydroxyproline in the soluble matrix. Enzyme-linked immunosorbent assay (ELISA) was used to detect the concentration of hydroxyproline and GAGs in the sample solution. Hydroxyproline occupies a specific proportion in collagen. Quantitative determination of hydroxyproline content is a common method for quantitative detection of collagen.

Evaluation of mechanical properties

Samples at 0-, 2- and 4-week culturing points were taken from each group and subjected to an unconfined compression test using a testing machine (ETM103A, Shenzhen Wantest Testing Equipment Co., Ltd.). The compression stroke was set to be within 1.5 mm vertically, and the compression speed was set to be 3 mm/min. The compressive modulus can be calculated via curve fitting of the stress–strain curve.

Results

The live/dead staining results for samples at 0-, 2- and 4-week culturing times are shown in Fig. 3. The chondrocytes were found to be evenly distributed in the samples of all groups, and the cells were in good condition at the beginning of the culture. After 2 and 4 weeks of culture, dead cells appeared for all groups, but could be neglectable compared to the large number of viable cells. The results also indicate that the chondrocytes always maintained a spherical shape in the calcium alginate scaffold and did not show significant differences in terms of the morphology or distribution among different groups.

Sections of samples at 10 μm thickness were prepared along the vertical plane in alignment with the rubbing direction after 4 weeks of culture, whose histological studying results are shown in Fig. 4 [25]. Each group of cells in the scaffolds grown well and the form of cell was normal. The cells in the sliding compression group (group S) and the single compression group (group P) showed some aggregation in the middle pores of the scaffold, whilst the cells in the control group and natural articular cartilage were relatively dispersed.

The cell proliferation for samples taken on the 2- and 4-week culturing time is examined and presented in Fig. 5. After 2 weeks of dynamic cultivation, the cell proliferation of all groups was increased significantly, and the proliferation rate of all dynamic culturing groups was higher than that of the static culturing control group (group C). After 4 weeks of culture, the cell proliferation rate of all groups was still significantly higher than that at the starting point, but the proliferation rate decreased compared with those of the second week, and the cell proliferation rate of dynamic groups was lower than that of the control group. The cell proliferation rate of the group with the frequency of 1 Hz (group S) was significantly higher than the other two groups with a frequency of 0.25 Hz or 0.5 Hz (group SF1 or group SF2).

At the end of the second and fourth weeks, the samples were digested using the method as described in "Remodelling of ECM" section, and the matrix was extracted and the content of GAGs and the collagen in the matrix is shown in Figs. 6 and 7. Results showed that the GAGs content in the dynamically cultured groups except the group SA1 and group SA2 were significantly higher than that of the control group. The content of GAGs in the group with a compression strain amplitude of 20% (group S) was significantly higher than that in the groups with a strain amplitude of 5% or 10% (group SA1 or SA2). The collagen content in all dynamic culturing groups was significantly higher than that in the control group, but there was no significant difference in the collagen content between individual dynamically culturing groups at the 4 weeks. The collagen content in the sliding compression group (group S) was higher than that in the simple compression group (group P).

The elastic modulus of the samples at 0, 2 and 4 weeks was derived from the stress–strain curve tested using the Mechanical Experiment Machine, as shown in Fig. 8. There is no difference observed in the elastic modulus of each group of samples at the week 0, so the measured elastic modulus value was taken as the initial elastic modulus. The elastic modulus of the specimens decreased with the culture time for all groups, and there was no significant difference between the various loading groups at the same examination point. The elastic modulus of the samples in dynamically culturing groups was lower than that of the control group.

Discussion

In this study, a method for preparing a calcium alginate hydrogel with a regular shape via crosslinking sodium of alginate with a calcium chloride solution at room temperature was developed. Young rabbit chondrocytes were isolated and seeded into the alginate gel to prepare the TEC specimen. The results showed that the chondrocytes in the scaffolds were in good condition and maintained a normal morphology, which confirmed the feasibility of the process for preparing TEC samples. A cartilage tissue biosimulator that could apply periodic vertical compression movement with horizontal reciprocating motion on the specimens was constructed, which realized coupling of compression,

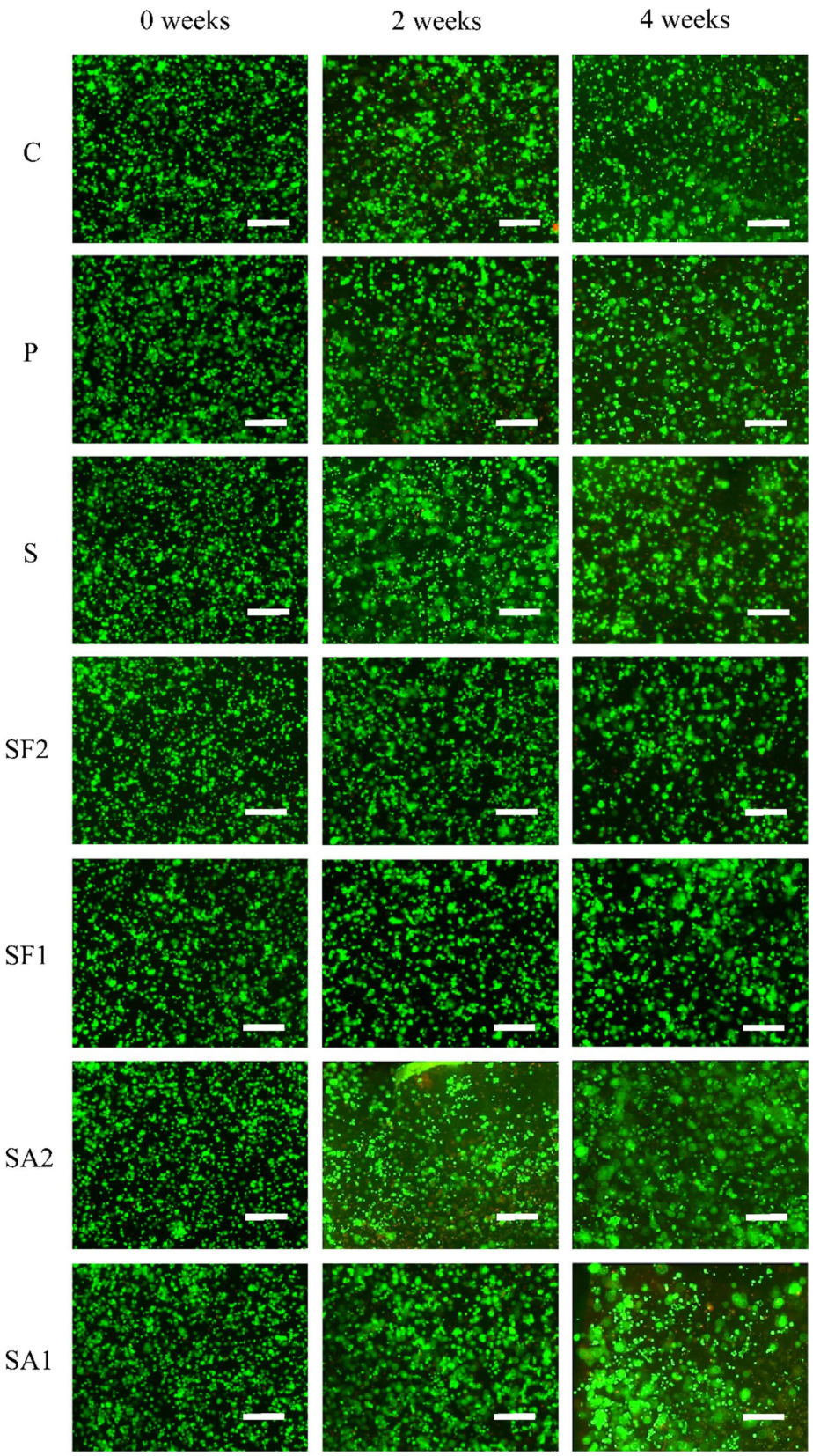

Fig. 3 Live/dead staining results for samples from different groups at 0, 2 and 4 weeks of culture time. Scale bar: 300 μm

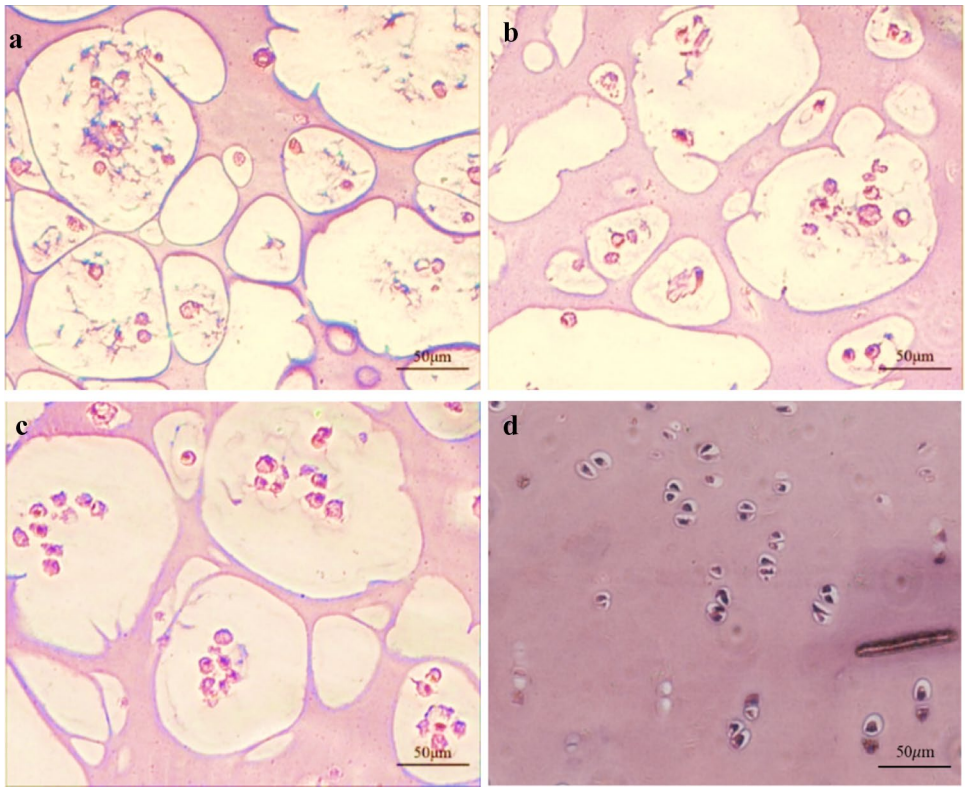

Fig. 4 H&E staining of tissue-engineered cartilage in **a** C, **b** P and **c** S groups, and **d** natural articular cartilage

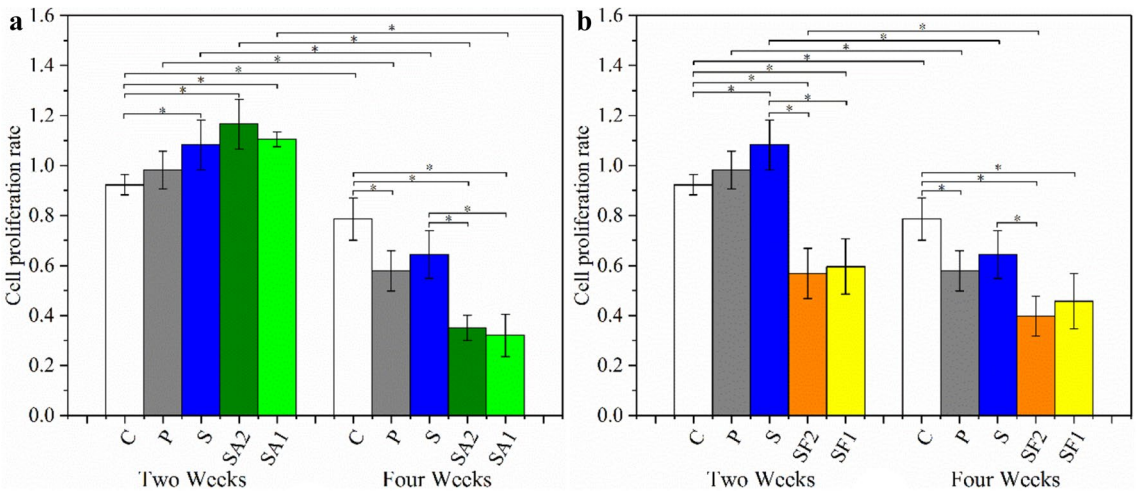

Fig. 5 Results of cell proliferation test (**a** Different compression amplitude groups; **b** different frequency groups) (*: one-way ANOVA, $p < 0.05$, $n = 4$)

friction and shearing mechanical stimulation. Moreover, the effects of the loading form (groups C, P and S), compressive strain amplitude (groups S, SA1, SA2 and C) and loading frequency (groups S, SF1, SF2 and C) on tissue engineering chondrocyte activity, cell proliferation, matrix remodelling and elastic modulus were evaluated.

In the tissue viability test, the dead and alive cell staining results at week 0 showed that the chondrocytes in all groups were uniformly distributed and in good condition, which confirmed the feasibility of the tissue-engineered cartilage preparation process used in this study. Although dead cells gradually appeared in each group at the second and fourth weeks, the living cells still accounted for the majority, which showed that the samples in each group survived well during the dynamic cultivation process. However, the chondrocytes did not spread in the calcium alginate scaffold and there

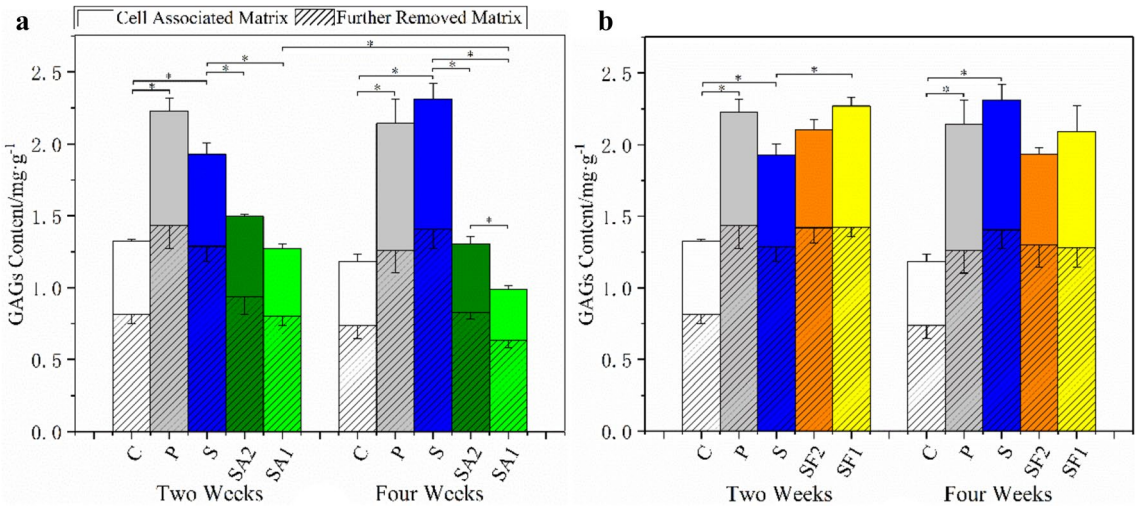

Fig. 6 Comparisons of GAGs content in different groups (**a** Different compression amplitude groups; **b** different frequency groups) (*: one-way ANOVA, $p<0.05$, $n=4$)

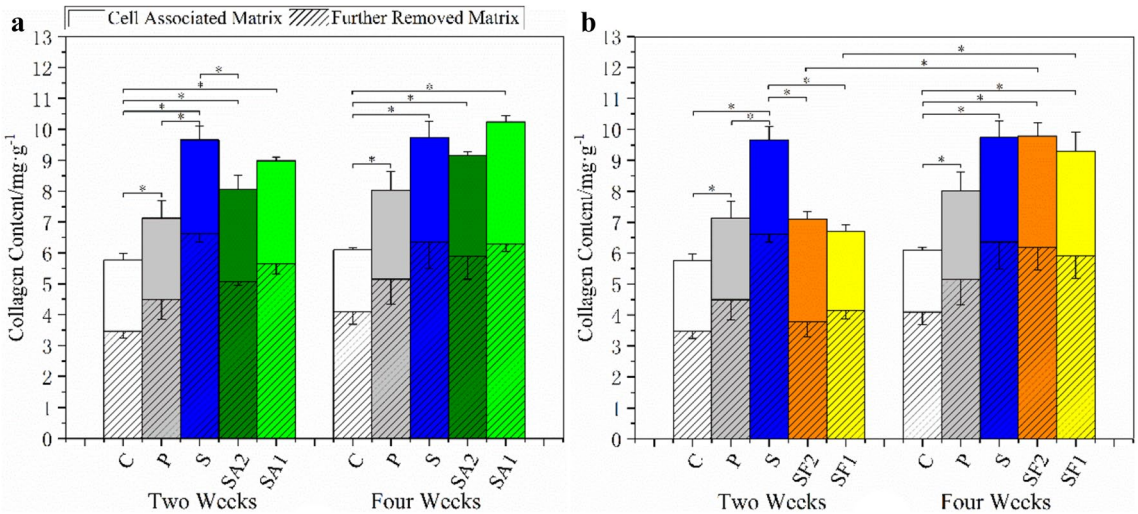

Fig. 7 Comparisons of collagen content in different groups (**a** Different compression amplitude groups; **b** different frequency groups) (*: one-way ANOVA, $p<0.05$, $n=4$)

were no significant differences between the control group (group C) and the experimental groups in the morphology and distribution of the cells.

The compression experiment results showed that there was not a positive correlation between the elastic modulus and the matrix content. The elastic modulus of the samples gradually decreased with time because the mechanical properties of tissue-engineered cartilage are primarily determined by the cellular scaffold and the calcium alginate polymer in the scaffolds was degraded. Another reason for the elastic modulus decrease was the lack of barrier protection between the macromolecules in the matrix and the culture medium, which allowed the matrix secreted by the chondrocytes to easily diffuse into the culture medium. Collagen fibres could not limit proteoglycan absorption of water and the consequent swelling. Therefore, the internal matrix content of the tissue-engineered cartilage increased and the mechanical properties did not improve as expected [29].

Static and dynamic compression loads were applied to the specimens to simulate the knee cartilage under standing and exercising conditions. The cell proliferation test results showed that dynamic mechanical stimulation in the first 2 weeks promoted the proliferation of seed cells, and the sliding compression was more favourable to the promotion of the cells. Group SA1 showed a higher proliferation ratio compared to group S at the second week. Thus, the group

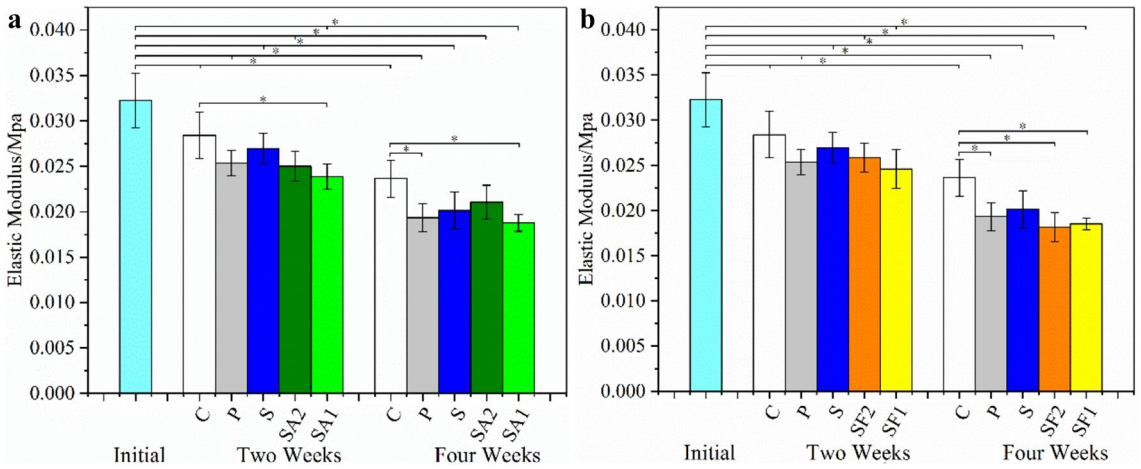

Fig. 8 Comparisons of elastic modulus in different groups (**a** Different compression amplitude groups; **b** different frequency groups) (*: one-way ANOVA, $p < 0.05$, $n = 4$)

S may have reached a higher peak before the second week, which might indicate that increasing the compressive strain amplitude might lead to a higher peak and also in lesser time. From the view of nutritional supply, dynamic compression and sliding compression both stimulated the flow of nutrient solution in the tissue-engineered cartilage. Therefore, the nutritional supply of tissue engineering cartilage in the experimental groups (groups P and S) was better than that in the static control group (group C). The number of cells in each group decreased from week 2 to week 4, which indicated that the number of cells in the tissue-engineered cartilage reached a peak value between week 0 and week 4. At 4 weeks, the number of cells in group P and group S was less than that in group C, and the reason might be that the time at which the peak number of cells was reached under mechanical stimulation was earlier than that in the static control group. Histological results showed that the chondrocytes of group P and group S were more likely to gather together, similar to what can be found in the natural cartilage tissue. This might indicate that mechanical action causes physical migration of cells, which was friendly with regard to the functionalization of the chondrocytes. The results of matrix content testing showed that dynamic mechanical stimulation promoted the secretion of GAGs and collagen by chondrocytes. By comparing the amount of GAG amount in groups P, S and C, it could be concluded that the dynamic compressive stress was the main mechanical factor regulating the secretion of GAGs in the matrix.

The effect of compression stress amplitude on cell proliferation in each group was significant. The primary chondrocytes used in this study cannot proliferate indefinitely and the cell proliferative ability decreased with time. Compared with high-amplitude (group S) dynamic compression, low-amplitude sliding compression (group SA1) could not effectively promote flow of the culture medium in three-dimensional cell scaffold during dynamic culture. Therefore, nutrient and metabolite exchange was blocked under the low-amplitude sliding compression situation, which resulted in the lowest cell proliferation rate in group SA1. Similarly, the secretion of GAGs in tissue-engineered cartilage also increased with increasing strain amplitude.

The change in loading frequency also had effects on cell proliferation. In the range of 0.25–1 Hz (groups SF1, SF2 and S), low-frequency sliding compression load was not conducive to cell proliferation. From the perspective of nutrient penetration, the high-frequency sliding compression load promoted entry of the culture medium into the scaffold, which was the main cause of the low cell proliferation rate in the lower-frequency group. Matrix content testing showed that sliding compression loads of different frequencies all promoted the secretion of GAGs, and there was no significant difference in the promotion of GAG secretion. However, the change in loading frequency did not affect collagen secretion until the fourth week.

Although this study quantified the effect of dynamic compressive load and shear stress on the secretion and elastic modulus of tissue-engineered cartilage from the perspective of the stress mode of the cartilage, it was still insufficient. The selection of calcium alginate as the scaffold material cannot avoid degradation of the scaffold material and the loss of the matrix secreted by the cartilage when the samples were cultured in vitro, which caused the mechanical properties of the calcium alginate scaffolds to decrease with time. In addition, the biosimulator built in this study cannot measure or provide feedback on the friction of the tissue in real time, nor could it apply a rolling compression load to the samples. These issues need to be improved in future studies.

Conclusion

The secretion of extracellular matrix in tissue-engineered cartilage is essential for regeneration and functional reconstruction of articular cartilage defects. A bespoke biosimulator was built to incorporate the coupled motion of compression, friction and shear. The results indicated that, in the range of 5–20% of the amplitude of strain and a frequency range of 0.25–1 Hz, a larger strain and higher frequency were more favourable for the specimen in terms of the tissue bioactivities and extracellular matrix synthesis. Moreover, the combined mechanical stimulation was more beneficial to matrix remodelling than the single loading motion. However, the contribution of the combined mechanical stimulation to the engineered cartilaginous tissue matrix was not sufficient to impede the biodegradation of the tissue with culture time.

Acknowledgements The work was supported by National Key R&D Program of China [2018YFE0207900], Key R&D Program of Guangdong Province [2018B090906001], the Fundamental Research Funds for the Central Universities and the Youth Innovation Team of Shaanxi Universities and the EU via the H2020-MSCA-RISE-2016 program [734156].

Author contributions ZH participated in the study design, experimental research, data analysis, writing and editing of the manuscript. SW, AF and JK performed the experimental research and data analysis. JN performed the experimental research, the study design and data analysis. DL and CL performed writing and editing of the manuscript. LW performed the study design, writing and editing of the manuscript. All authors have read and approved the final manuscript and, therefore, have full access to all the data in the study and take responsibility for the integrity and security of the data.

Availability of data and material The materials used in the article are purchased through regular channels, and the experimental data were true and reliable.

Compliance with ethical standards

Conflicts of interest The authors declare that they have no conflict of interest.

Ethical approval This research has been approved by the Animal Management Committee of Xi'an Jiaotong University.

Consent to participate All authors agree to participate in the work related to this article.

Consent for publication The manuscript was approved by all authors for publication.

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