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RESEARCH ARTICLE

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Conditioned medium of mesenchymal stem cells delays osteoarthritis progression in a rat model by protecting subchondral bone, maintaining matrix homeostasis, and enhancing autophagy

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Abstract

Evidence accumulated that mesenchymal stem cell (MSC) therapy ameliorated osteoarthritis (OA) via paracrine effect, whereas conditioned medium (CM) of MSCs contains all the secretomes. In vitro studies have proved its therapeutic effect in OA, but few in vivo evidences were unveiled. This study investigated the effect of MSCs–CM in an animal model of OA. OA was induced by anterior cruciate ligament transaction and destabilization of the medial meniscus in 12 rats bilaterally. The CM group (N = 6) was administered with intraarticular injection of MSCs–CM weekly, whereas the phosphate-buffered saline (PBS) group (N = 6) was injected with PBS. Six rats served as normal control and received sham operation with weekly PBS injection. Rats were sacrificed 8 weeks postoperatively. Gross and histological morphology were analysed. Microcomputed tomography was applied to assess the subchondral bone. Components of extracellular matrix (ECM) including type II collagen (Col II) and aggrecan, and ECM homeostasis-related enzymes (metalloproteinase-13 [MMP-13] and tissue inhibitor of metalloproteinase-1 [TIMP-1]), as well as autophagy markers (Beclin-1 and microtubule-associated protein light chain 3) were evaluated immunohistochemically. Chondrocyte apoptosis was measured by terminal deoxynucleotidyl transferase dUTP nick-end labelling staining. Gene expression of Col II, aggrecan, MMP-13, and TIMP-1 was confirmed by real-time polymerase chain reaction. Morphological outcomes demonstrated remarkable articular-protective effect of MSCs–CM. Well-maintained subchondral bone structure, significantly more abundant cartilage matrix, notably decreased ratio of MMP-13 to TIMP-1, and inhibited chondrocyte apoptosis with enhanced autophagy were observed in the CM group compared with the PBS group. In conclusion, MSCs–CM demonstrated satisfactory effect in alleviating OA in rats via protecting the microarchitecture of subchondral bone, balancing the ratio of MMP-13 to TIMP-1 in cartilage, and enhancing autophagy, which might provide a new remedy against OA.

*co-first authors and contributed equally to this work.

KEYWORDS

articular cartilage, autophagy, conditioned medium, MSCs, osteoarthritis, subchondral bone

1 | INTRODUCTION

Osteoarthritis (OA), the most prevalent chronic disease menacing articular cartilage, has been predicted to be the fourth biggest culprit of disability in 2020 (Toh, Lai, Hui, & Lim, 2017). Inflammation, apoptosis of chondrocytes and loss of cartilage extracellular matrix (ECM) are the hallmarks of OA progression, leading to degradation of articular cartilage (Mi et al., 2018).

Currently, modalities that can reverse the progression of OA are still being developed. Nonsteroidal anti-inflammatory drugs and corticosteroids merely relieve pain temporarily but have limited effect on preventing chondrocyte apoptosis and matrix degradation (Wang, Yu, Liu, et al., 2017). What is more, according to the guideline from OA Research Society International published in 2014, the effect of intraarticular injection of hyaluronic acid, oral administration of glucosamine, and physiotherapies such as acupuncture and electrotherapy were uncertain even in symptom relief of OA and were not appropriate for disease modification (McAlindon et al., 2014). Moreover, except arthroplasty for end-stage patients, surgical interventions such as mosaicplasty, microfracture, and autologous chondrocyte implantation not only induce donor site morbidity but also regenerate inferior fibrocartilage (Jiang, Zhang, Qi, Wang, & Ouyang, 2011). Hence, it is of great value to develop a remedy to circumvent the pathological process.

In the recent years, mesenchymal stem/stromal cell-based therapy (MSCT) has become a promising way in treating OA. Several studies proved the effect of MSCT in cartilage repair in animal models and even clinical studies (de Windt et al., 2017; Hsu et al., 2018; Koh, Kwon, Kim, Choi, & Tak, 2016; Xia et al., 2018). However, concerning the risk of tumourigenicity (Deng, Zhang, Xie, Zhang, & Tang, 2018), genomic stability, and immunogenicity when using viable cells (Zhang et al., 2013), there is still a long way before popularization of MSCT in clinical practice. Besides, the whole process from manufacturing qualified cells to maintaining their viability until delivering to patients is costly (Zhang et al., 2016), which might put a considerable burden on the sufferers.

On the other hand, a number of studies used cell tracking technique to find that only limited amount of implanted mesenchymal stem cells (MSCs) differentiated into regenerated cartilage (Matsumoto et al., 2009; Murphy, Fink, Hunziker, & Barry, 2003). Moreover, evidence accumulated that it is the trophic factors secreted by MSCs, including cytokines, growth factors, chemokines (Meirelles, Fontes, Covas, & Caplan, 2009), microvesicles (Bruno et al., 2009), exosomes (Lai et al., 2010), and ECM (Lee, Kwon, Lee, Son, & Chung, 2017), should take the credit in tissue repair. Considering all these secretions are contained in MSCs' culture medium, also known as conditioned medium (CM; Soleimanifar et al., 2018), it is a safer choice to use cell-free CM as a substitute for MSCT. Increasing publications have demonstrated the effectiveness of cell-free CM in ameliorating high glucose-induced degradation of ECM (Qi et al., 2018), inflammatory arthritis (Kay et al., 2017), wound healing (Amini et al., 2018), and spinal cord injury (Kanekiyo et al., 2018). Considering the regenerative property of CM, we therefore measured the potential of CM against OA in a rat model. We hypothesize that intraarticularly injected CM can alleviate the progression of OA in the rat models.

In this study, OA models were created by anterior cruciate ligament transection and destabilization of the medial meniscus because anterior cruciate ligament tears and meniscus injuries are two of the main causes of OA in real life (Wang, Yu, Liu, et al., 2017). The CM of human bone marrow-derived stem cells (hBMSCs) was intraarticularly injected once a week from the first day after the surgery. We evaluated the gross and histological morphology, the subchondral bone architecture, the haemostasis of ECM, and the apoptosis and autophagy of chondrocytes in rats.

2 | MATERIALS AND METHODS

2.1 | Culture of human bone marrow-derived stem cells

Passage 0 hBMSCs purchased from ScienCell Research Laboratories (CA, USA) were cultured in Mesenchymal Stem Cell Medium (ScienCell Research Laboratories, CA, USA) using a humidified incubator at 5% CO_2 atmosphere and 37°C. At the point when the confluency of passage 3 hBMSCs reached 70% in 75-cm^2 flasks with complete medium, wash the cells three times with phosphate-buffered saline (PBS) and refed with 10-ml serum-free Dulbecco's modified Eagle's medium without antibiotics for 48 hr. Collect the supernatant, centrifuge it twice at 1,500 rpm for 10 min to remove cellular debris, and then store it at −80°C till use.

2.2 | Animal study design

This study was approved by the Institutional Animal Care and Use Committee and carried out following the Guide for the Care and Use of Laboratory Animals. Eighteen 12-week-old male Sprague Dawley rats-(280–320 g) were used in the study. First, 12 rats were randomly picked out and received bilateral anterior cruciate ligament transection and destabilization of the medial meniscus according to the previous study (C. J. Wang, Cheng, Huang, et al., 2017), whereas the remaining six rats and 12 legs received sham arthrotomy. The 12 with surgeries were randomly divided into two groups, namely, PBS group (PBS) and CM group (CM), and the six with sham surgeries were normal control group (normal). For the PBS and normal groups, rats received intraarticular injection of 100-μl PBS weekly started from the first

day after surgery, whereas the CM group was administered with 100-μl CM in the same way. The needle was inserted at the middle of the patellar tendon at a 30° angle from the tibia. Rats could move freely in their cages and get enough food and water ad libitum.

2.3 | Gross and histology morphology

The animals were euthanized by CO_2 overdose at 8 weeks postoperatively, and the knee specimens were collected and analysed. The gross morphology of articular cartilage of femurs and tibias was inspected under a magnification scope (Carl Zeiss, Oberkochen, Germany) and scored following the protocol of a previous study (C. J. Wang, Cheng, Chou, et al., 2017) by a blinded observer. The specimens were fixed in 10% formalin for 72 hr and decalcified in EDTA solution. After decalcification, they were dehydrated and buried in paraffin. Five-micrometre sections were chopped and stained with haematoxylin and eosin and Safranin O/Fast Green (Saf-O) to observe the histological morphology and the deposition of sulfated glycosaminoglycan (s-GAG). The Saf-O outcomes were scored by modified Mankin score (Li, Cai, Zhang, Cui, & Shen, 2015).

2.4 | Microcomputed tomography analysis

The subchondral bones of the knees were evaluated using microcomputed tomography (Skyscan 1076 scanner; Skyscan, Aartselaar, Belgium). The voxel size was 18 μm. The X-ray voltage was 70 kV, and a 1.0-mm aluminium plate was used as filter. Rotation step was set as 0.8° with full width. NRecon software (Skyscan) was used to reconstruct the image slices. The photographs of the coronal planes of the knees were shot by DataViewer software (Skyscan). Bone volume fraction (BV/TV, %), trabecular number (Tb.N, mm^{-1}), and trabecular separation (Tb.Sp, mm) of every tibia and femur were analysed by CT-Analyser Software (Skyscan).

2.5 | Immunohistochemistry and immunofluorescence

Immunohistochemical staining was conducted following a standard protocol (Y. Wang, Yu, Liu, et al., 2017). Cartilage matrix was detected using type II collagen (COL II) and aggrecan. Metalloproteinase-13 (MMP-13) and tissue inhibitor of metalloproteinase-1 (TIMP-1) were examined to evaluate the homeostasis of ECM, whereas Beclin-1 and microtubule-associated protein light chain 3 were observed to appraise autophagy of chondrocytes. The percentage of positive cells of each biomarker and the ratio of MMP-13 to TIMP-1 positive cells (MMP-13/TIMP-1) were calculated for quantitative analysis. Besides, immunofluorescence was utilized to detect apoptosis by using terminal deoxynucleotidyl transferase dUTP nick-end labelling (TUNEL) assay according to a previous research article (Dai, Sui, Xue, Liu, & Sun, 2018). Images were observed by fluorescence microscopy (Olympus IX73, Japan). The expression level of positive cells was measured by ImageJ Version 1.51t.

2.6 | Real-time polymerase chain reaction

Articular cartilage was harvested from the femoral condyles after the section and then grounded by a mortar and pestle in liquid nitrogen. TRIzol reagent (Invitrogen, Carlsbad, CA, USA) was utilized to isolate total RNA from the samples following manufacturer's instructions. Took 1 μg of total RNA from each sample, and reverse transcribed them by iScript™ reverse transcription Supermix (Bio-Rad Laboratories, Hercules, CA, USA), and use CFX Connect™ real-time PCR system (Bio-Rad) with iTaq™ Universal SYBR® Green Supermix (Bio-Rad) to amplify. The primers were presented in Table 1. The cycling condition of polymerase chain reaction included a 30-s initial denaturation at 95°C following 40 cycles of amplification. The amplification step comprised denaturation at 95°C of 15 s and extension at 60°C of 30s. Glyceraldehyde 3-phosphate dehydrogenase mRNA was used as reference gene, and the results were calculated using the comparative $^{\triangle}$CT method.

2.7 | Statistical analysis

All quantitative statistics were expressed as mean ± standard error of the mean. SPSS 16.0 software (SPSS Inc., Chicago, USA) was used to analyse the data. Student's *t* test or one-way analysis of variance was utilized to calculate the statistical difference between groups, followed by Bonferroni post hoc analysis if significance was present. Significant difference was set as p value <.05.

3 | RESULTS

3.1 | Gross morphology

As shown in Figure 1a, the articular cartilage of the PBS group appeared as a coarse surface full of fissures with osteophyte formation and subchondral bone exposed compared with that of the normal group with limited fibrillations. By contrast, the articular surface in the

TABLE 1 Real-time PCR primer sequences

Gene	Primer sequence
COL2A1	F: 5′-CCCCTGCAGTACATGCGG-3′ R: 5′-CTCGACGTCATGCTGTCTCAAG-3′
Aggrecan	F: 5′-CAGTGCGATGCAGGCTGGCT-3′ R: 5′-CCTCCGGCACTCGTTGGCTG-3′
TIMP-1	F: 5′-GACCACCTTATACCAGCGTT-3′ R: 5′-GTCACTCTCCAGTTTGCAAG-3′
MMP-13	F: 5′-TCGCATTGTGAGAGTCATGCCAACA-3′ R: 5′-TGTGGTTCCAGCCACGCATAGTCA-3′
GAPDH	F: 5′-GGTCGGTGTGAACGGATTTGG-3′ R: 5′-GCCGTGGGTAGAGTCATACTGGAAC-3′

Abbreviations: GAPDH, glyceraldehyde 3-phosphate dehydrogenase; MMP-13, metalloproteinase-13; PCR, polymerase chain reaction; TIMP-1, tissue inhibitor of metalloproteinase-1.

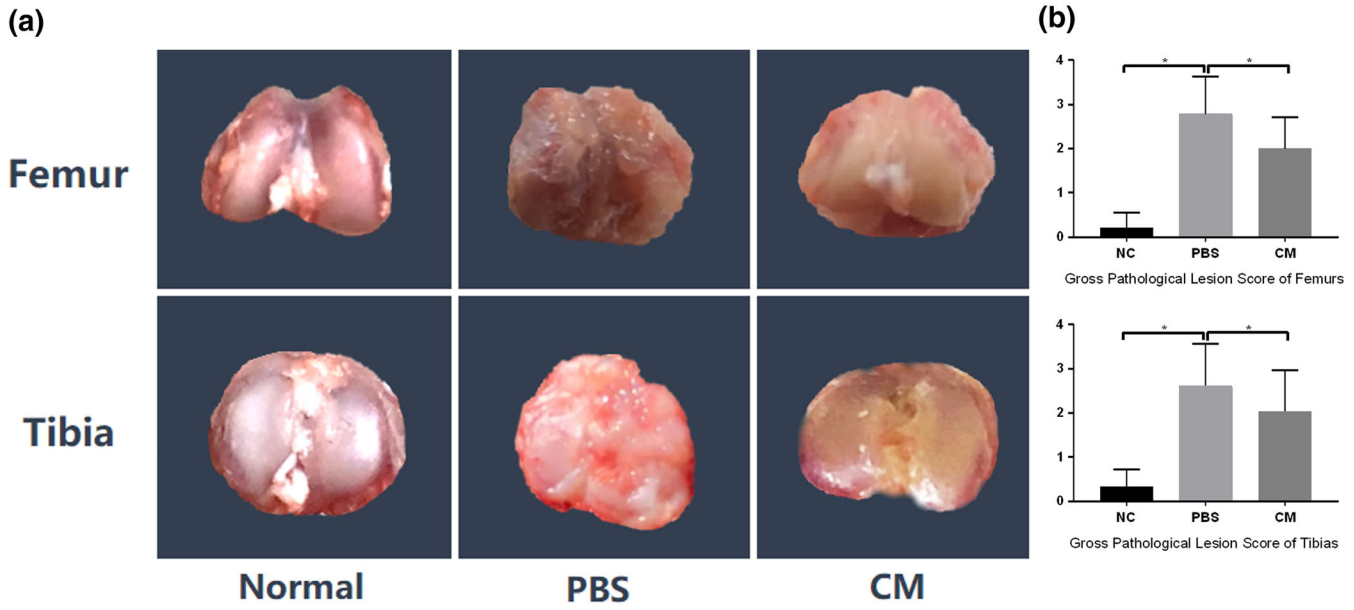

FIGURE 1 Gross morphology 8 weeks postoperatively. (a) Representative photos of articular surface. (b) The gross pathological lesion scores. Data were measured as mean ± standard error of the mean. $^{*}p < .05$ compared with the phosphate-buffered saline (PBS) group. CM, conditioned medium; NC, normal control [Colour figure can be viewed at wileyonlinelibrary.com]

CM group was much smoother. The protective effect of CM on cartilage was also affirmed by gross pathological lesion score (Figure 1b).

3.2 | Histological morphology

The articular cartilage improvement in the CM group was further confirmed in the histological assessment. As revealed by haematoxylin

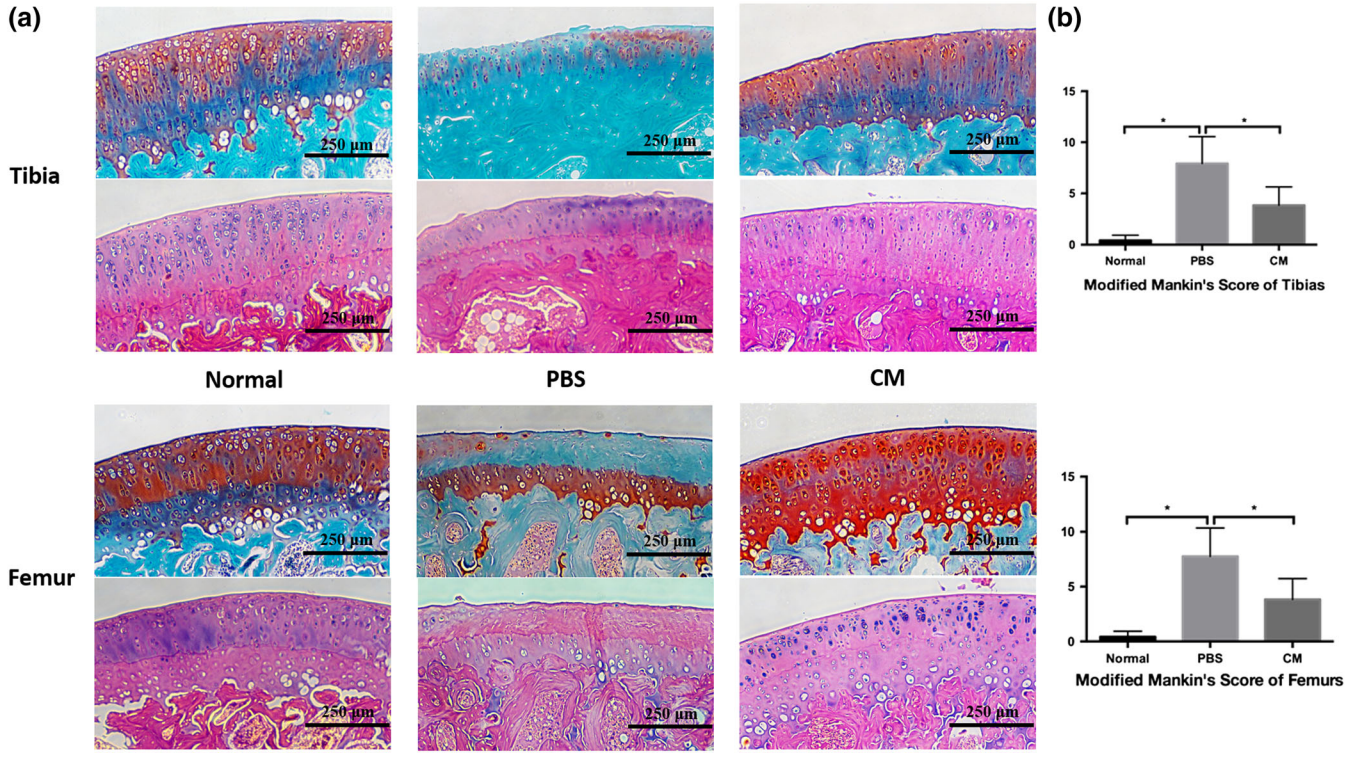

FIGURE 2 Histological morphology 8 weeks postoperatively. (a) Representative photos of medial tibial plateau and femoral condyle from the midsagittal plane stained with Safranin O/Fast Green (top row) and haematoxylin and eosin (bottom row). (b) The modified Mankin's scores. Data were measured as mean ± standard error of the mean. $^{*}p < .05$ compared with the phosphate-buffered saline (PBS) group. CM, conditioned medium [Colour figure can be viewed at wileyonlinelibrary.com]

and eosin staining, the cartilage thickness and the number of chondrocytes significantly decreased with a ragged edge in the PBS group, whereas in the CM group, the cartilage thickness and surface regularity was comparable with the normal group, although the number of chondrocytes and the amount of blue staining of cartilage matrix remained lower than that of normal cartilage. Moreover, the Saf-O staining demonstrated that the s-GAG, an essential component of cartilage matrix, greatly degraded in the PBS group compared with rats treated with CM at both femoral and tibial sides (Figure 2a). The modified Mankin score was significantly higher in the PBS group than in the two other groups, indicating a histological improvement of cartilage deterioration by CM (Figure 2b).

3.3 | Subchondral bone changes

As presented in Figure 3a, the subchondral bone in the PBS group showed remarkable loss of bone volume, thickening, and deformation of the contour and destruction of the trabecular bone structure. However, in joints injected with CM, there was only limited loss of trabecular bone. BV/TV and Tb. N significantly decreased in both tibias and femurs of the PBS group compared with the CM and normal groups (Figure 3b,c). However, the Tb. Sp was significantly higher in tibias and femurs of the PBS group (Figure 3d).

3.4 | CM protected the cartilage matrix components

COL II and s-GAG are principal components of cartilage matrix (Zhang et al., 2018), and aggrecan constitutes about 90% of s-GAG in cartilage (Little & Fosang, 2010), making them ideal markers of cartilage status. Immunohistochemically, significantly stronger COL II as well as aggrecan positive staining was observed in CM-treated cartilage than the PBS group (Figure 4a–d). This finding was supported by the outcome of real-time polymerase chain reaction (RT-PCR), suggesting that the gene expression of both COL2A1 and aggrecan was significantly higher in the CM group than in the PBS group (Figure 4e,f).

3.5 | CM maintained the balance between MMP-13 and TIMP-1

The matrix of cartilage was modulated by enzymes and their inhibitors through a cascade of events. Among them, MMP-13 is a crucial enzyme that is implicated in degrading ECM of cartilage, whereas TIMP-1 is the specific inhibitor of MMP-13 (Onitsuka et al., 2018). In the PBS group, the MMP-13 level, as suggested by the percentage of positive cells after immunohistochemical staining, is significantly higher than the intact joints and joints treated with CM (Figure 5a,b), whereas the TIMP-1 level was remarkably increased in the CM group compared with the two other groups (Figure 5c,d), making MMP-13/TIMP-1 significantly lower in the CM group than in the PBS group (Figure 5e). Additionally, the outcome of RT-PCR tallied with that of immunohistochemistry (Figure 5f,g). These findings suggested that CM ameliorated OA-related ECM degradation.

3.6 | CM reduced the apoptosis of chondrocytes

Apoptosis of chondrocytes is another feature of the progression of OA (Bijlsma, Berenbaum, & Lafeber, 2011). By immunofluorescence, TUNEL, one of the most commonly used staining of cellular apoptosis, was applied. We observed a notable decrease of the proportion of TUNEL positive cells in cartilage of the CM group compared with the PBS group, whereas negligible expression of TUNEL was detected in the normal cartilage (Figure 6a). And the statistical significance was

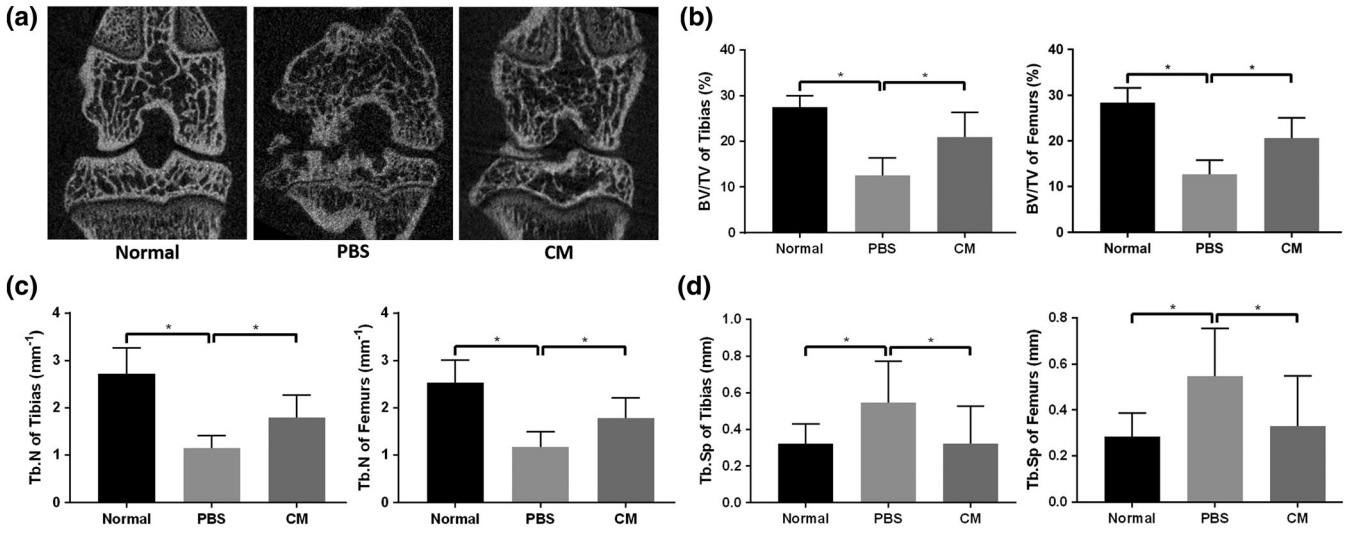

FIGURE 3 Microcomputed tomography scan of subchondral bone structure and relevant morphometric parameters of tibias and femurs 8 weeks postoperatively. (a) Representative photos of knee joints in coronal view from microcomputed tomography. (b) The BV/TV values from the region of interest in tibias and femurs. (c) The trabecular number (Tb.N) values from the region of interest in tibias and femurs. (d) The trabecular separation (Tb.Sp) values from the region of interest in tibias and femurs. Data were measured as mean ± standard error of the mean. $^{*}p < .05$ compared with the phosphate-buffered saline (PBS) group. BV/TV, bone volume fraction; CM, conditioned medium

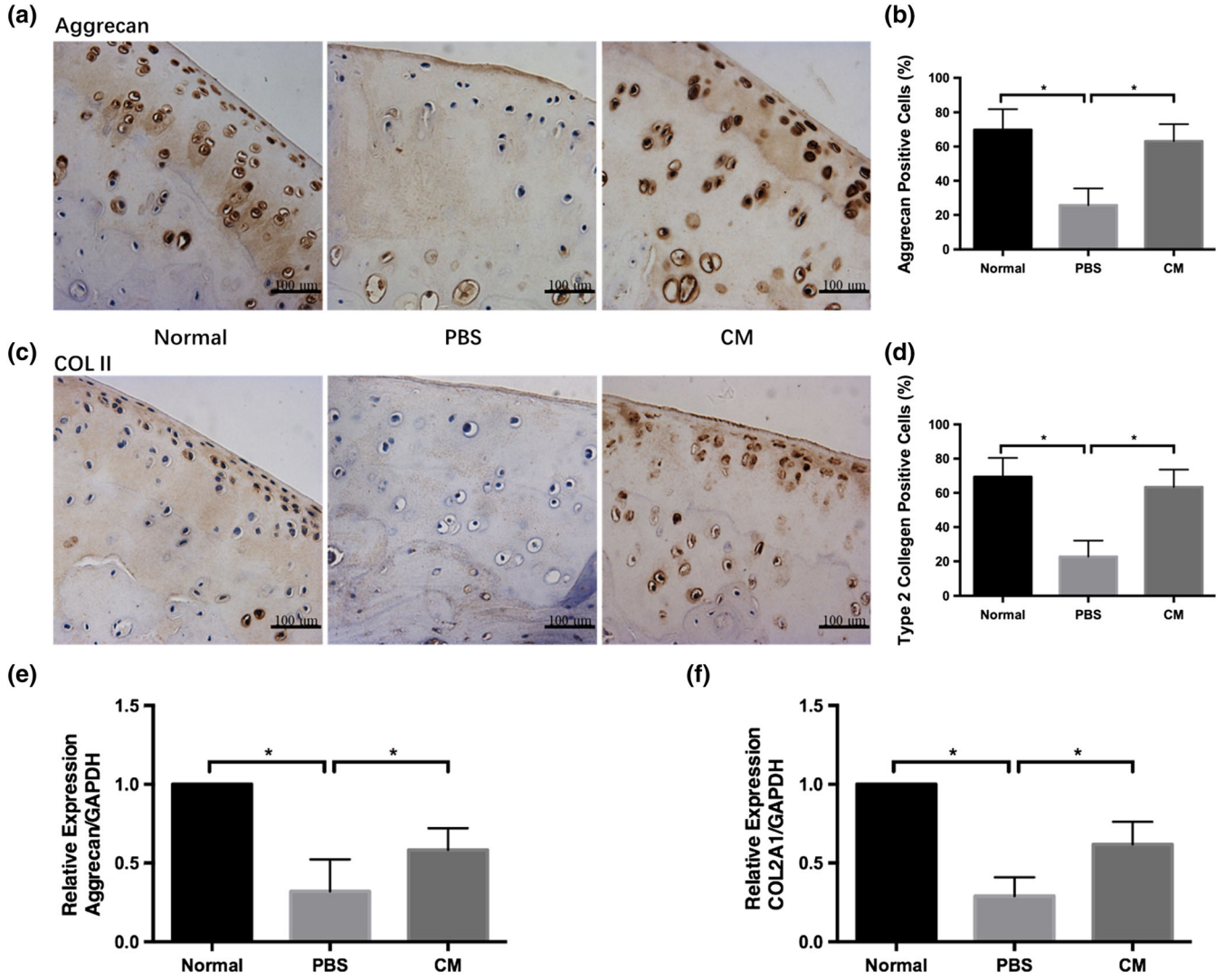

FIGURE 4 Cartilage matrix components 8 weeks postoperatively. (a) Representative photos of immunohistochemical staining of aggrecan. (b) The percentage of aggrecan positive cells. (c) Representative photos of immunohistochemical staining of type 2 collagen (COL II). (d) The percentage of COL II positive cells. (e) Real-time polymerase chain reaction analysis of aggrecan in rat primary chondrocytes. (f) Real-time polymerase chain reaction analysis of Col2a1 in rat primary chondrocytes. Data were measured as mean ± standard error of the mean. $^{*}p < .05$ compared with the phosphate-buffered saline (PBS) group. CM, conditioned medium; GAPDH, glyceraldehyde 3-phosphate dehydrogenase [Colour figure can be viewed at wileyonlinelibrary.com]

further verified by the quantitative analysis (Figure 6b), supporting that CM significantly reduced apoptosis under OA environment.

3.7 | CM activated autophagy in chondrocytes

Autophagy serves as an adaptive response under stress to promote survival of cells by preserving energy level in cells (Carames, Taniguchi, Otsuki, Blanco, & Lotz, 2010); therefore, we questioned whether the difference in TUNEL positive cells was related to autophagy. By detecting the level of Beclin-1 and microtubule-associated protein light chain 3, two vital proteins in the autophagic process, a dramatically reduced autophagic activity was detected in the PBS group than in the normal group, whereas the CM group maintained almost the same level as that of the normal group (Figure 6c,f). Taken together, our findings indicated that inhibition of chondrocyte apoptosis by promoting autophagy might also account for the protective role of MSCs–CM in OA.

4 | DISCUSSION

To our knowledge, this is the first study confirming the effects of MSCs–CM in the rat model of OA. We set two control groups, normal and PBS control, to eliminate the impacts from variations of inflammation cytokines in articular cavity caused by injection. According to our experiments in vivo, intraarticular administration of MSCs–CM significantly improved both gross and histology appearance of articular cartilage in contrast to PBS, verifying the previous findings from in vitro studies. We further discovered that the

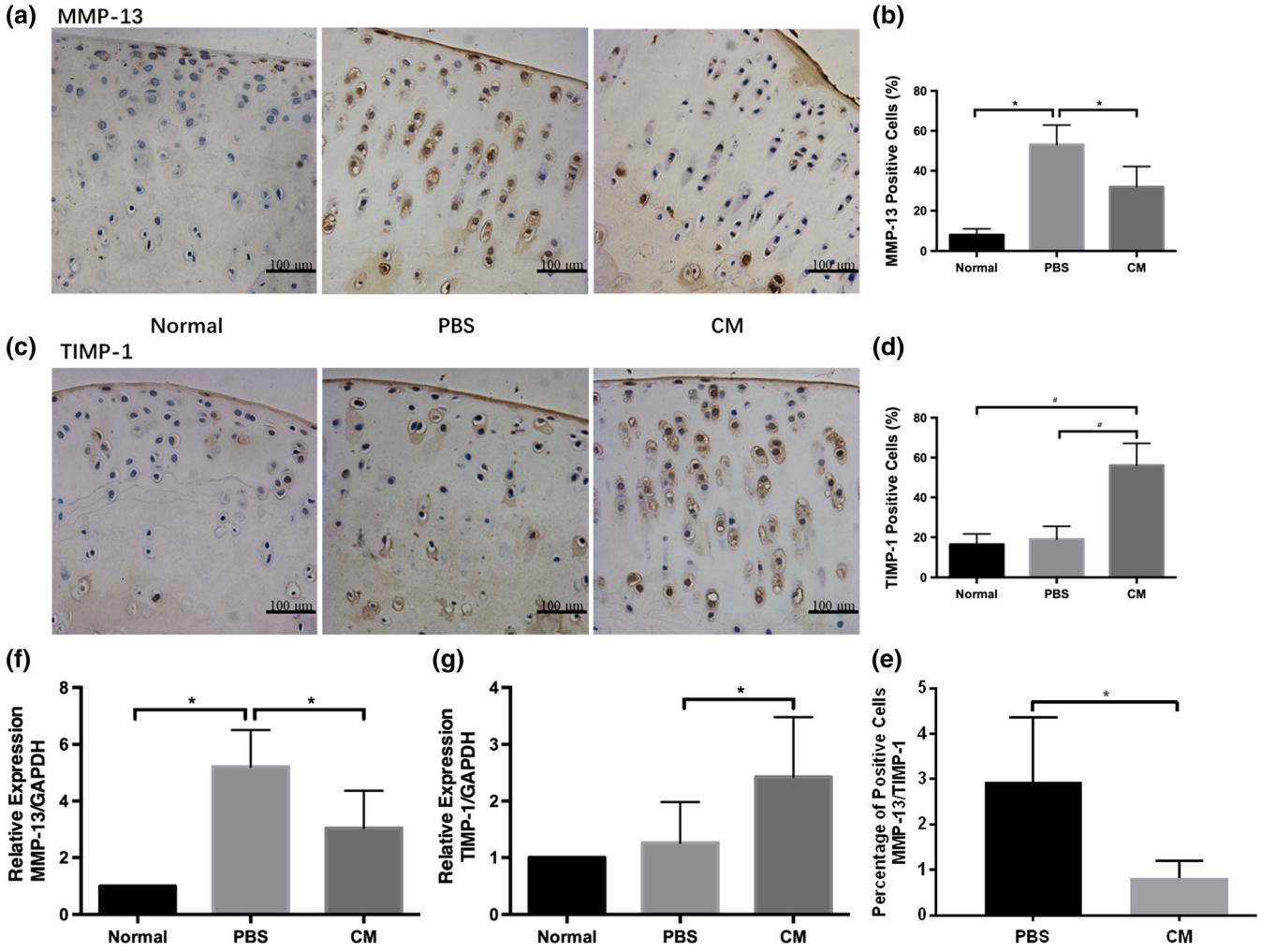

FIGURE 5 Expression level of metalloproteinase-13 (MMP-13) and tissue inhibitor of metalloproteinase-1 (TIMP-1) 8 weeks postoperatively. (a) Representative photos of immunohistochemical staining of MMP-13. (b) The percentage of MMP-13 positive cells. (c) Representative photos of immunohistochemical staining of TIMP-1. (d) The percentage of TIMP-1 positive cells. (e) Ratio of the percentage of MMP-13 positive cells to the percentage of TIMP-1 positive cells. (f) Real-time polymerase chain reaction analysis of MMP-13 in rat primary chondrocytes. (g) Real-time polymerase chain reaction analysis of TIMP-1 in rat primary chondrocytes. Data were measured as mean ± standard error of the mean. $^{*}p < .05$ compared with the phosphate-buffered saline (PBS) group; $^{\#}p < .05$ compared with the conditioned medium (CM) group. GAPDH, glyceraldehyde 3-phosphate dehydrogenase [Colour figure can be viewed at wileyonlinelibrary.com]

OA restraining effect of MSCs–CM was correlated with the well-protected subchondral bone structure, the regulation of enzymes that modulate cartilage matrix and the autophagy of chondrocytes that inhibits apoptosis, which indicates a potential pharmaceutical therapy that is safe, economical, and easy to produce to impede the progression of OA.

The attention on OA has been growing, ever since the emergence of aging problem, obesity problem, and the increasing frequency of sports injuries in the modern society. The progression of OA is characterized by degradation of articular cartilage as well as the remodelling of subchondral bone, which results in activity-related pain, swelling, stiffness, and movement limitation of the impaired joints, undermining patients' quality of life (Bijlsma et al., 2011). To overcome the influence of OA, in the United States alone, over two hundred billion dollars were invested annually (Neogi & Zhang, 2013), whereas there is still no valid nonsurgical therapy to alter the course of the disease.

For the past decade, MSCT has been a budding therapy to deal with various physical disorders due to the extraordinary self-renewal capacity and the multilineage differentiation potential of MSCs. In 2003, Murphy et al. firstly reported the administration of adult MSCs intraarticularly in an animal OA model, and such treatment promoted the meniscus regeneration and hindered the progressive destruction of articular cartilage. In 2008, the first case report of MSCT by Centeno et al. demonstrated improved cartilage regeneration and knee mobility together with significant pain reduction in an OA patient. Recently, the 2-year follow-up outcome of first-in-human prospective cohort study of intraarticular injection of MSCs treating OA by Jo et al. (2017) further confirmed the safety and efficacy of this procedure. Despite all that, the concerns regarding to the risk of cell-based therapy cannot be fully eliminated. Zhang et al. (2013) reported significant immune rejection in rats without neonatal desensitization after MSC injection treating cartilage defects. Additionally, in 2015,

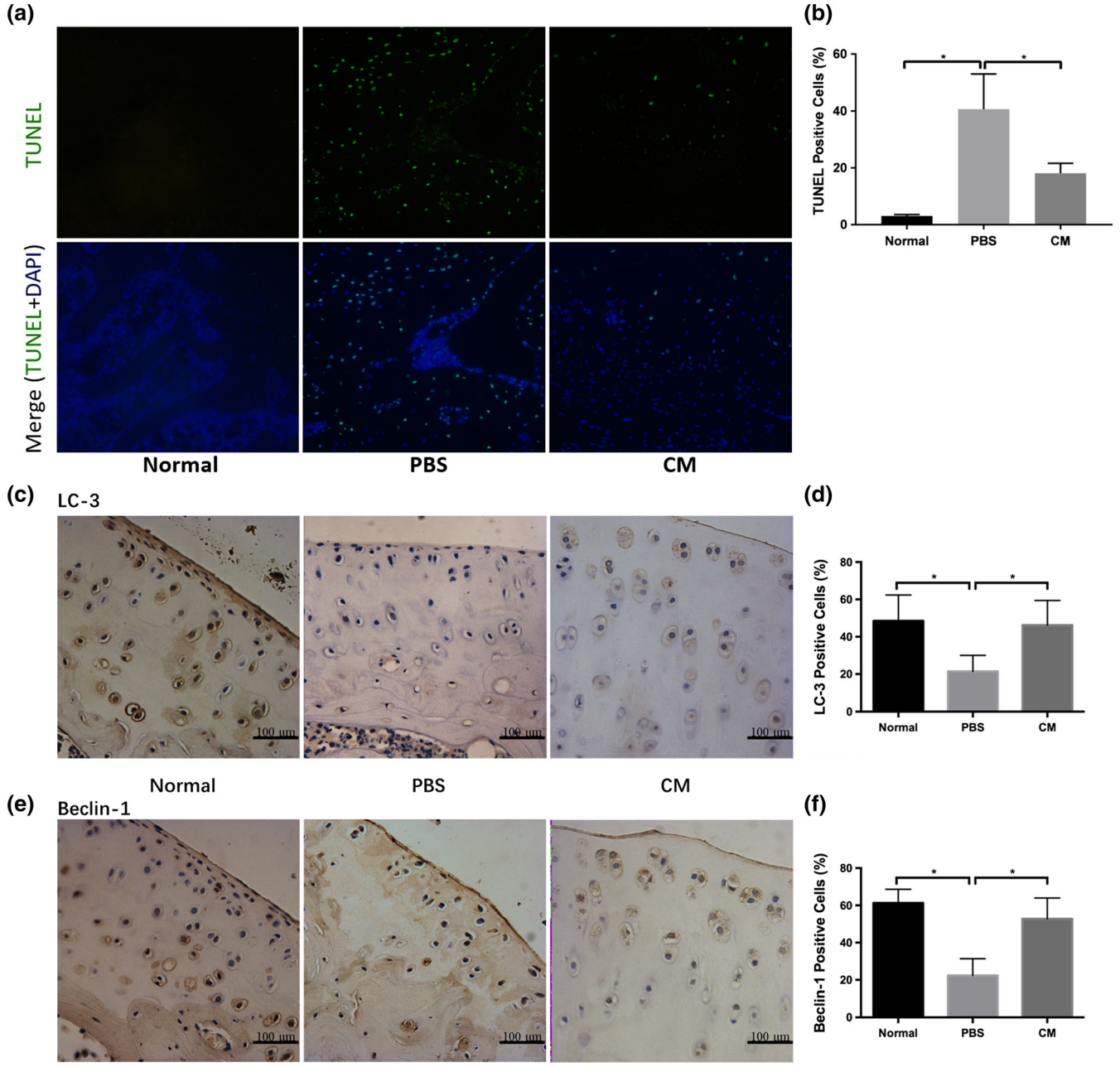

FIGURE 6 Chondrocyte apoptosis and autophagy 8 weeks postoperatively. (a) Representative photos of terminal deoxynucleotidyl transferase dUTP nick-end labelling (TUNEL; green) staining. (b) The percentage of TUNEL positive cells. Data were measured as mean ± standard error of the mean. (c) Representative photos of immunohistochemical staining of microtubule-associated protein light chain 3 (LC-3). (d) The percentage of LC-3 positive cells. (e) Representative photos of immunohistochemical staining of Beclin-1. (f) The percentage of Beclin-1 positive cells. Data were measured as mean ± standard error of the mean. $^{*}p < .05$ compared with the phosphate-buffered saline (PBS) group. CM, conditioned medium; DAPI, 4′,6-diamidino-2-phenylindole [Colour figure can be viewed at wileyonlinelibrary.com]

Nori et al. observed tumour formation derived from grafted stem cells in a mouse model of spinal cord injury treated with pluripotent stem cells. These issues mentioned above must be settled before MSCT becoming a routine procedure in clinic. And to top it all off, evidence revealed that scarcely, any transplanted cells were transformed into chondrocytes at the involved area as expected (Matsumoto et al., 2009; Murphy et al., 2003), which inspired researchers to realize the importance of paracrine effects of MSCs, including exosomes, microvesicles, growth factors, cytokines, and chemokines in the therapeutic process. This theory was supported by several in vitro studies. In 2012, van Buul et al. demonstrated that human synovium and cartilage explants cultured in CM of MSCs expressed less inflammatory and cartilage degradation-related gene compared with those in control medium. And Platas et al. (2013) revealed that the chondroprotective effects of CM were probably contributed by the inhibition of nuclear factor κB pathway. However, very few in vivo studies were published, which motivated our interests in testing MSCs–CM in an animal model of OA.

The microarchitecture of subchondral bone, mainly determined by its trabeculae arisen from subchondral bone plate, plays an essential role as a shock absorber and a buttress of a knee (Li et al., 2013). Remodulation of subchondral bone has long been regarded as one of the key contributing factors of cartilage degradation. In 1986, Radin and Rose put forth that the remodelling makes subchondral bone denser and less compliant generating shear stress to destruct the cartilage above. In the present study, subchondral bone was evaluated via microcomputed tomography, and the PBS group showed massive bone destruction as well as obvious thickening and irregularity of the edge. Besides, the trabecular bone microarchitecture also deteriorated, suggested by the analyses of morphometric parameters. Therefore, the comparable manifestations in the CM and normal groups indicated a subchondral bone-protective effect of MSCs–CM (Li et al., 2013). Hayami et al. (2006) verified that the bone resorption inhibitor could effectively protect articular cartilage in the rat model of OA. Then in 2015, Chang et al. observed the osteogenesis potent of MSCs–CM in healing bone defects. Hence, it is possible that the osteogenesis potent of CM maintained the balance between resorption and formation of subchondral bone, which further preserved the microarchitecture of subchondral bone.

Additionally, ECM, which accounts for 95% of the volume of articular cartilage, is another crucial target in OA treatment. Composed of collagen and proteoglycan, ECM functions as load supporter and shelters chondrocytes from excessive stresses (Felson et al., 2000). The collagens line parallel to the articular surface to withstand shear stress, whereas proteoglycans draw water and salt ions to resist the compressive load (Bhosale & Richardson, 2008). In the current study, the immunohistochemistry outcome together with RT-PCR doubly confirmed the ECM-protective effect of CM. The expression of COL II and aggrecan, predominant components of cartilage ECM, was comparable in the CM and normal groups but significantly decreased in the PBS group, which enlightened us to measure the expression of enzymes responsible for ECM homeostasis and find the alteration of the ratio of MMP-13 to TIMP-1 in cartilage after injection of MSCs–CM. For decades, excessive expression of MMP-13 during OA progression has been regarded as the primary cause of ECM degradation, whereas TIMP-1 is the inhibitor of MMP-13 that can bind to the metalloproteinase at a 1:1 molar ratio to hinder the enzyme activity (Onitsuka et al., 2018). As observed by Shiraishi et al. (2017), MMPs/TIMPs significantly increased in ex vivo OA cartilage, but the homeostasis could be recovered by the paracrine effect of human adipose tissue-derived stem cells, which was consistent with our study. On ground of this, the protection of cartilage by CM was possibly the sequel of equilibrium between MMP-13 and TIMP-1.

The ECM is primarily synthesized and secreted by chondrocytes (Toh et al., 2017), which means the intensified apoptosis of chondrocytes in OA milieu contributes the loss of ECM (Rahmati, Nalesso, Mobasheri, & Mozafari, 2017). The results of immunofluorescence in our study revealed significantly lower expression of TUNEL in CM-treated joints compared with the PBS group, indicating anti-apoptosis effect of CM. Shiraishi et al. (2017) also noticed this potency of paracrine from human adipose tissue-derived stem cells, whereas the mechanism underneath is yet to be clarified. In our study, we examined the biomarker of autophagy, which is a self-protective cellular mechanism against apoptosis when exposed to stress stimuli by utilizing lysosomal enzyme to hydrolyse impaired or dysfunctional macromolecules and organelles. In 2010, Carames et al. discovered autophagy as a protective mechanism in normal cartilage but was hampered by OA, resulting in cartilage degradation. Since then, the importance of autophagy in OA therapy has been proved repeatedly (Meng et al., 2018). Furthermore, Tsai et al. reported that MSCs–CM ameliorated spinal cord injury by promoting autophagy. Similarly, in this study, MSCs–CM was likely to delay the progression of OA by enhancing autophagy to reduce the apoptosis of chondrocytes, which further improved the synthesis and expression of proteoglycan and COL II and sustained the homeostasis of ECM, a necessity for articular cartilage to withstand daily mechanical stresses.

Although this study presented the promising potent of MSCs–CM in delaying the progression of OA, there were still limitations in this study. First, only one timepoint was set in the study so that the dynamic changes of cartilage degradation as well as subchondral bone destruction cannot be observed. Second, rats are not upright walking animals, which means larger animal like sheep with knee joint's moving pattern more resembling that of human beings has to be used in preclinical experiment before applying of MSCs–CM in clinic. Third, the specific role and mechanism of each constituent in MSCs–CM during this therapeutic process have not been thoroughly elucidated, which can orient our future researches.

In conclusion, intraarticular injection of MSCs–CM demonstrated a satisfactory effect in alleviating OA in a rat model via protecting the microarchitecture of subchondral bone, balancing the ratio of MMP-13 to TIMP-1 in cartilage to maintain the homeostasis of ECM, and enhancing autophagy to compete chondrocyte apoptosis. Considering its safety and economy compared with cell-based therapy, it might provide a new remedy to cope with OA in the future.

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CONFLICT OF INTEREST

None.

AUTHOR CONTRIBUTIONS

W. C., Y. S., and X. G. contributed equally to this study. W. C. and Y. S. performed the experiments and wrote the manuscript. X. G. analysed

most of the data and plotted the figures. X. L. performed the micro-CT and analysed the relevant data. J. L. participated in some of the experiments. Y. H. and J. C. reviewed the manuscript. S. C. organized the work and reviewed the manuscript.

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REFERENCES

Amini, A., Pouriran, R., Abdollahifar, M. A., Abbaszadeh, H. A., Ghoreishi, S. K., Chien, S., & Bayat, M. (2018). Stereological and molecular studies on the combined effects of photobiomodulation and human bone marrow mesenchymal stem cell conditioned medium on wound healing in diabetic rats. *Journal of Photochemistry and Photobiology. B*, *182*, 42–51. https://doi.org/10.1016/j.jphotobiol.2018.03.010

Bhosale, A. M., & Richardson, J. B. (2008). Articular cartilage: Structure, injuries and review of management. *British Medical Bulletin*, *87*, 77–95. https://doi.org/10.1093/bmb/ldn025

Bijlsma, J. W., Berenbaum, F., & Lafeber, F. P. (2011). Osteoarthritis: An update with relevance for clinical practice. *Lancet*, *377*(9783), 2115–2126. https://doi.org/10.1016/S0140-6736(11)60243-2

Bruno, S., Grange, C., Deregibus, M. C., Calogero, R. A., Saviozzi, S., Collino, F., … Camussi, G. (2009). Mesenchymal stem cell-derived microvesicles protect against acute tubular injury. *Journal of the American Society of Nephrology*, *20*(5), 1053–1067. https://doi.org/10.1681/ASN.2008070798

Carames, B., Taniguchi, N., Otsuki, S., Blanco, F. J., & Lotz, M. (2010). Autophagy is a protective mechanism in normal cartilage, and its aging-related loss is linked with cell death and osteoarthritis. *Arthritis and Rheumatism*, *62*(3), 791–801. https://doi.org/10.1002/art.27305

Centeno, C. J., Busse, D., Kisiday, J., Keohan, C., Freeman, M., & Karli, D. (2008). Increased knee cartilage volume in degenerative joint disease using percutaneously implanted, autologous mesenchymal stem cells. *Pain Physician*, *11*(3), 343–353.

Chang, W., Kim, R., Park, S. I., Jung, Y. J., Ham, O., Lee, J., … Maeng, L. S. (2015). Enhanced healing of rat calvarial bone defects with hypoxic conditioned medium from mesenchymal stem cells through increased endogenous stem cell migration via regulation of ICAM-1 targeted-microRNA-221. *Molecules and Cells*, *38*(7), 643–650. https://doi.org/10.14348/molcells.2015.0050

Dai, M., Sui, B., Xue, Y., Liu, X., & Sun, J. (2018). Cartilage repair in degenerative osteoarthritis mediated by squid type II collagen via immunomodulating activation of M2 macrophages, inhibiting apoptosis and hypertrophy of chondrocytes. *Biomaterials*, *180*, 91–103. https://doi.org/10.1016/j.biomaterials.2018.07.011

de Windt, T. S., Vonk, L. A., Slaper-Cortenbach, I. C., van den Broek, M. P., Nizak, R., van Rijen, M. H., … Saris, D. B. (2017). Allogeneic mesenchymal stem cells stimulate cartilage regeneration and are safe for single-stage cartilage repair in humans upon mixture with recycled autologous chondrons. *Stem Cells*, *35*(1), 256–264. https://doi.org/10.1002/stem.2475

Deng, J., Zhang, Y., Xie, Y., Zhang, L., & Tang, P. (2018). Cell transplantation for spinal cord injury: Tumorigenicity of induced pluripotent stem cell-derived neural stem/progenitor cells. *Stem Cells International*, *2018*, 5653787. https://doi.org/10.1155/2018/5653787

Felson, D. T., Lawrence, R. C., Dieppe, P. A., Hirsch, R., Helmick, C. G., Jordan, J. M., … Fries, J. F. (2000). Osteoarthritis: New insights. Part 1: The disease and its risk factors. *Annals of Internal Medicine*, *133*(8), 635–646. https://doi.org/10.7326/0003-4819-133-8-200010170-00016

Hayami, T., Pickarski, M., Zhuo, Y., Wesolowski, G. A., Rodan, G. A., & Duong, L. T. (2006). Characterization of articular cartilage and subchondral bone changes in the rat anterior cruciate ligament transection and meniscectomized models of osteoarthritis. *Bone*, *38*(2), 234–243. https://doi.org/10.1016/j.bone.2005.08.007

Hsu, Y. K., Sheu, S. Y., Wang, C. Y., Chuang, M. H., Chung, P. C., Luo, Y. S., … Kuo, T. F. (2018). The effect of adipose-derived mesenchymal stem cells and chondrocytes with platelet-rich fibrin releasates augmentation by intra-articular injection on acute osteochondral defects in a rabbit model. *The Knee*, *25*(6), 1181–1191. https://doi.org/10.1016/j.knee.2018.10.005

Jiang, Y. Z., Zhang, S. F., Qi, Y. Y., Wang, L. L., & Ouyang, H. W. (2011). Cell transplantation for articular cartilage defects: Principles of past, present, and future practice. *Cell Transplantation*, *20*(5), 593–607. https://doi.org/10.3727/096368910X532738

Jo, C. H., Chai, J. W., Jeong, E. C., Oh, S., Shin, J. S., Shim, H., & Yoon, K. S. (2017). Intra-articular injection of mesenchymal stem cells for the treatment of osteoarthritis of the knee: A 2-year follow-up study. *The American Journal of Sports Medicine*, *45*(12), 2774–2783. https://doi.org/10.1177/0363546517716641

Kanekiyo, K., Wakabayashi, T., Nakano, N., Yamada, Y., Tamachi, M., Suzuki, Y., … Ide, C. (2018). Effects of intrathecal injection of the conditioned medium from bone marrow stromal cells on spinal cord injury in rats. *Journal of Neurotrauma*, *35*(3), 521–532. https://doi.org/10.1089/neu.2017.5201

Kay, A. G., Long, G., Tyler, G., Stefan, A., Broadfoot, S. J., Piccinini, A. M., … Kehoe, O. (2017). Mesenchymal stem cell-conditioned medium reduces disease severity and immune responses in inflammatory arthritis. *Scientific Reports*, *7*(1), 18019. https://doi.org/10.1038/s41598-017-18144-w

Koh, Y. G., Kwon, O. R., Kim, Y. S., Choi, Y. J., & Tak, D. H. (2016). Adipose-derived mesenchymal stem cells with microfracture versus microfracture alone: 2-year follow-up of a prospective randomized trial. *Arthroscopy*, *32*(1), 97–109. https://doi.org/10.1016/j.arthro.2015.09.010

Lai, R. C., Arslan, F., Lee, M. M., Sze, N. S., Choo, A., Chen, T. S., … Lim, S. K. (2010). Exosome secreted by MSC reduces myocardial ischemia/reperfusion injury. *Stem Cell Research*, *4*(3), 214–222. https://doi.org/10.1016/j.scr.2009.12.003

Lee, S. Y., Kwon, B., Lee, K., Son, Y. H., & Chung, S. G. (2017). Therapeutic mechanisms of human adipose-derived mesenchymal stem cells in a rat tendon injury model. *The American Journal of Sports Medicine*, *45*(6), 1429–1439. https://doi.org/10.1177/0363546517689874

Li, G., Yin, J., Gao, J., Cheng, T. S., Pavlos, N. J., Zhang, C., & Zheng, M. H. (2013). Subchondral bone in osteoarthritis: Insight into risk factors and microstructural changes. *Arthritis Research & Therapy*, *15*(6), 223. https://doi.org/10.1186/ar4405

Li, W., Cai, L., Zhang, Y., Cui, L., & Shen, G. (2015). Intra-articular resveratrol injection prevents osteoarthritis progression in a mouse model by activating SIRT1 and thereby silencing HIF-2α. *Journal of Orthopaedic Research*, *33*(7), 1061–1070. https://doi.org/10.1002/jor.22859

Little, C. B., & Fosang, A. J. (2010). Is cartilage matrix breakdown an appropriate therapeutic target in osteoarthritis—Insights from studies of aggrecan and collagen proteolysis? *Current Drug Targets*, *11*(5), 561–575. https://doi.org/10.2174/138945010791011956

Matsumoto, T., Cooper, G. M., Gharaibeh, B., Meszaros, L. B., Li, G., Usas, A., … Huard, J. (2009). Cartilage repair in a rat model of osteoarthritis through intraarticular transplantation of muscle-derived stem cells expressing bone morphogenetic protein 4 and soluble Flt-1. *Arthritis and Rheumatism*, *60*(5), 1390–1405. https://doi.org/10.1002/art.24443

McAlindon, T. E., Bannuru, R. R., Sullivan, M. C., Arden, N. K., Berenbaum, F., Bierma-Zeinstra, S. M., … Underwood, M. (2014). OARSI guidelines for the non-surgical management of knee osteoarthritis. *Osteoarthritis and Cartilage*, *22*(3), 363–388. https://doi.org/10.1016/j.joca.2014.01.003

Meirelles, L. S., Fontes, A. M., Covas, D. T., & Caplan, A. I. (2009). Mechanisms involved in the therapeutic properties of mesenchymal stem cells. *Cytokine & Growth Factor Reviews*, *20*(5-6), 419–427. https://doi.org/10.1016/j.cytogfr.2009.10.002

Meng, Z., Shen, B., Gu, Y., Wu, Z., Yao, J., Bian, Y., … Zhao, Y. (2018). Diazoxide ameliorates severity of experimental osteoarthritis by activating autophagy via modulation of the osteoarthritis-related biomarkers. *Journal of Cellular Biochemistry*, *119*(11), 8922–8936. https://doi.org/10.1002/jcb.27145

Mi, B., Wang, J., Liu, Y., Liu, J., Hu, L., Panayi, A. C., … Zhou, W. (2018). Icariin activates autophagy via down-regulation of the NF-κB signaling-mediated apoptosis in chondrocytes. *Frontiers in Pharmacology*, *9*, 605. https://doi.org/10.3389/fphar.2018.00605

Murphy, J. M., Fink, D. J., Hunziker, E. B., & Barry, F. P. (2003). Stem cell therapy in a caprine model of osteoarthritis. *Arthritis and Rheumatism*, *48*(12), 3464–3474. https://doi.org/10.1002/art.11365

Neogi, T., & Zhang, Y. (2013). Epidemiology of osteoarthritis. *Rheumatic Diseases Clinics of North America*, *39*(1), 1–19. https://doi.org/10.1016/j.rdc.2012.10.004

Nori, S., Okada, Y., Nishimura, S., Sasaki, T., Itakura, G., Kobayashi, Y., … Okano, H. (2015). Long-term safety issues of iPSC-based cell therapy in a spinal cord injury model: Oncogenic transformation with epithelial–mesenchymal transition. *Stem Cell Reports*, *4*(3), 360–373. https://doi.org/10.1016/j.stemcr.2015.01.006

Onitsuka, K., Murata, K., Kokubun, T., Fujiwara, S., Nakajima, A., Morishita, Y., & Kanemura, N. (2018). Effects of controlling abnormal joint movement on expression of MMP13 and TIMP-1 in osteoarthritis. *Cartilage*, 941195303. https://doi.org/10.1177/1947603518783449

Platas, J., Guillen, M. I., Del, C. M., Gomar, F., Mirabet, V., & Alcaraz, M. J. (2013). Conditioned media from adipose-tissue-derived mesenchymal stem cells down regulate degradative mediators induced by interleukin-1 beta in osteoarthritic chondrocytes. *Mediators of Inflammation*, *2013*, 357014. https://doi.org/10.1155/2013/357014

Qi, L., Wang, R., Shi, Q., Yuan, M., Jin, M., & Li, D. (2018). Umbilical cord mesenchymal stem cell conditioned medium restored the expression of collagen II and aggrecan in nucleus pulposus mesenchymal stem cells exposed to high glucose. *Journal of Bone and Mineral Metabolism*, *37*, 455–466. https://doi.org/10.1007/s00774-018-0953-9

Radin, E. L., & Rose, R. M. (1986). Role of subchondral bone in the initiation and progression of cartilage damage. *Clinical Orthopaedics and Related Research*, (213), 34–40. PMID: 3780104.

Rahmati, M., Nalesso, G., Mobasheri, A., & Mozafari, M. (2017). Aging and osteoarthritis: Central role of the extracellular matrix. *Ageing Research Reviews*, *40*, 20–30. https://doi.org/10.1016/j.arr.2017.07.004

Shiraishi, K., Kamei, N., Takeuchi, S., Yanada, S., Mera, H., Wakitani, S., … Ochi, M. (2017). Quality evaluation of human bone marrow mesenchymal stem cells for cartilage repair. *Stem Cells International*, *2017*, 8740294. https://doi.org/10.1155/2017/8740294

Soleimanifar, F., Hosseini, F. S., Atabati, H., Behdari, A., Kabiri, L., Enderami, S. E., … Saburi, E. (2018). Adipose-derived stem cells-conditioned medium improved osteogenic differentiation of induced pluripotent stem cells when grown on polycaprolactone nanofibers. *Journal of Cellular Physiology*, *234*, 10315–10323. https://doi.org/10.1002/jcp.27697

Toh, W. S., Lai, R. C., Hui, J., & Lim, S. K. (2017). MSC exosome as a cell-free MSC therapy for cartilage regeneration: Implications for osteoarthritis treatment. *Seminars in Cell & Developmental Biology*, *67*, 56–64. https://doi.org/10.1016/j.semcdb.2016.11.008

van Buul, G. M., Villafuertes, E., Bos, P. K., Waarsing, J. H., Kops, N., Narcisi, R., … van Osch, G. J. (2012). Mesenchymal stem cells secrete factors that inhibit inflammatory processes in short-term osteoarthritic synovium and cartilage explant culture. *Osteoarthritis and Cartilage*, *20*(10), 1186–1196. https://doi.org/10.1016/j.joca.2012.06.003

Wang, C. J., Cheng, J. H., Chou, W. Y., Hsu, S. L., Chen, J. H., & Huang, C. Y. (2017). Changes of articular cartilage and subchondral bone after extracorporeal shockwave therapy in osteoarthritis of the knee. *International Journal of Medical Sciences*, *14*(3), 213–223. https://doi.org/10.7150/ijms.17469

Wang, C. J., Cheng, J. H., Huang, C. Y., Hsu, S. L., Lee, F. Y., & Yip, H. K. (2017). Medial tibial subchondral bone is the key target for extracorporeal shockwave therapy in early osteoarthritis of the knee. *American Journal of Translational Research*, *9*(4), 1720–1731.

Wang, Y., Yu, D., Liu, Z., Zhou, F., Dai, J., Wu, B., … Liu, H. (2017). Exosomes from embryonic mesenchymal stem cells alleviate osteoarthritis through balancing synthesis and degradation of cartilage extracellular matrix. *Stem Cell Research & Therapy*, *8*(1), 189. https://doi.org/10.1186/s13287-017-0632-0

Xia, T., Yu, F., Zhang, K., Wu, Z., Shi, D., Teng, H., … Jiang, Q. (2018). The effectiveness of allogeneic mesenchymal stem cells therapy for knee osteoarthritis in pigs. *Annals of Translational Medicine*, *6*(20), 404. https://doi.org/10.21037/atm.2018.09.55

Zhang, S., Chu, W. C., Lai, R. C., Lim, S. K., Hui, J. H., & Toh, W. S. (2016). Exosomes derived from human embryonic mesenchymal stem cells promote osteochondral regeneration. *Osteoarthritis and Cartilage*, *24*(12), 2135–2140. https://doi.org/10.1016/j.joca.2016.06.022

Zhang, S., Chuah, S. J., Lai, R. C., Hui, J., Lim, S. K., & Toh, W. S. (2018). MSC exosomes mediate cartilage repair by enhancing proliferation, attenuating apoptosis and modulating immune reactivity. *Biomaterials*, *156*, 16–27. https://doi.org/10.1016/j.biomaterials.2017.11.028

Zhang, S., Jiang, Y. Z., Zhang, W., Chen, L., Tong, T., Liu, W., … Zou, X. (2013). Neonatal desensitization supports long-term survival and functional integration of human embryonic stem cell-derived mesenchymal stem cells in rat joint cartilage without immunosuppression. *Stem Cells and Development*, *22*(1), 90–101. https://doi.org/10.1089/scd.2012.0116

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