Parathyroid hormone (1-34) promotes the effects of 3D printed scaffold-seeded bone marrow mesenchymal stem cells on meniscus regeneration

CURRENT STATUS: UNDER REVIEW

Wen Zhao
Northwest Agriculture and Forestry University

Tong Zou
Northwest Agriculture and Forestry University

Hao Cui
Northwest Agriculture and Forestry University

Yangou Lv
Northwest Agriculture and Forestry University

Dengke Gao
Northwest Agriculture and Forestry University

Chenmei Ruan
Northwest Agriculture and Forestry University

Xia Zhang
Northwest Agriculture and Forestry University

Yihua Zhang
Northwest Agriculture and Forestry University

zyh19620207@163.com Corresponding Author
ORCID: https://orcid.org/0000-0002-2939-5187

SUBJECT AREAS
- Stem Cell & Developmental Cell Biology
- Endocrinology & Metabolism

KEYWORDS
- Tissue engineering, meniscus, BMSCs, PTH (1-34), articular cartilage
Abstract
Background
Cell-based tissue engineering represents a promising management for meniscus repair and regeneration. The present study aimed to investigate whether the injection of parathyroid hormone (PTH) (1–34) could promote the regeneration and chondroprotection of 3D printed scaffold seeded with bone marrow mesenchymal stem cells (BMSCs) in a canine total meniscal meniscectomy model.

Methods
3D printed poly(e-caprolactone) scaffold seeded with BMSCs was cultured in vitro, and the effects of in vitro culture time on cell growth and matrix synthesis of the BMSCs-scaffold construct were evaluated by microscopic observation and cartilage matrix content detection at 7, 14, 21, and 28 days. After that, the tissue-engineered meniscus based on BMSCs-scaffold cultured for the appropriate culture time was selected for in vivo implantation. Sixteen dogs were randomly divided into four groups: PTH + BMSCs-scaffold, BMSCs-scaffold, total meniscectomy, and sham operation. The regeneration of the implanted tissue and the degeneration of articular cartilage were assessed by gross, histological, and immunohistochemical analysis at 12 weeks postoperatively.

Results
In vitro study showed that the glycosaminoglycan (GAG)/DNA ratio and the expression of collagen type II (Col2) were significantly higher on day 21 as compared to the other time points. In vivo study showed that, compared with the BMSCs-scaffold group, the PTH + BMSCs-scaffold group showed better regeneration of the implanted tissue and greater similarity to native meniscus with respect to gross appearance, cells composition, and cartilage extracellular matrix deposition. This group also showed less expression of terminal differentiation markers of BMSC chondrogenesis as well as lower cartilage degeneration with less damage on the knee cartilage surface, higher expression of Col2 and lower expression of degeneration markers.

Conclusions
Our results demonstrated that PTH (1–34) promotes the regenerative and chondroprotective effects of the BMSCs–3D printed meniscal scaffold in a canine model, and thus their combination could be a promising strategy for meniscus tissue engineering.

Background
Meniscus injury severely limits knee function and increase the risk of osteoarthritis[1]. In recent years, the rapid development in the fields of tissue engineering and regenerative medicine has provided a promising treatment for meniscus injury[2, 3]. The main steps involved in meniscus tissue engineering are preparing a scaffold and seeding cells and regulating the cell–scaffold construct through cytokines, mechanical stimulation, and other methods in order to synthesize the extracellular matrix (ECM) in vitro, followed by its transplantation in vivo for meniscus regeneration and function[4]. 3D printing technology can fabricate scaffolds with complete control of size, shape, and porosity; it has been used in many previous studies to prepare tissue-engineered meniscus scaffolds[5–8]. Bone marrow mesenchymal stem cells (BMSCs) are easy to isolate and proliferate, have low immunogenicity, and potential to differentiate into cartilage; thus, they have become an ideal seed cells for meniscus tissue engineering[9, 10]. However, the available literature does not answer several problems related to their use.

First, the accumulation of a certain amount of ECM in the in vitro culture is conducive to the better functioning of the cell–scaffold construct in response to knee pressure [11]. However, the influence of the in vitro culture time on the growth and differentiation of seed cells in meniscus tissue engineering has not been determined. Therefore, in order to select the appropriate implantation time, it is necessary to determine the effects of in vitro culture time on the cell growth and ECM accumulation on cell–scaffold constructs. Second, BMSCs often undergo terminal differentiation during chondrogenesis, which hampers the regenerative efficacy of tissue engineering[12, 13]; this limitation needs to be addressed when using BMSCs as seed cells for meniscus tissue engineering. Furthermore, although tissue-engineered meniscus transplantation can promote the recovery of knee joint function, it also causes different degrees of damage to knee cartilage and subchondral bone[14, 15].

Parathyroid hormone (PTH) (1–34) inhibits the hypertrophy of BMSCs during chondrogenic differentiation[16, 17], inhibits articular cartilage degeneration, and promotes articular chondrocyte proliferation and ECM synthesis[18]. Therefore, it is often used in studies on cartilage tissue engineering as well as in osteoarthritis treatment[19–21]. Orth et al.[22] reported that injection of PTH (1–34) into a rabbit model of osteochondral defect significantly increased the expression of PTH
receptor in chondrocytes and osteocytes in the rabbit joint, improved the surface structure and integration of articular cartilage, and reconstructed the subchondral bone. Zhang et al.[23] combined Parathyroid hormone related protein (PTHrP) treatment with collagen–silk scaffold implantation and found that it improved the osteochondral defect repair efficacy. However, to the best of our knowledge, there are currently no reports on the repair of meniscus defects by combining PTH (1–34) and tissue-engineered meniscus.

In this study, we aimed to determine the appropriate in vitro culture time for 3D printed meniscus scaffolds seeded with canine BMSCs. Further, we transplanted these into meniscectomy model dogs to investigate the repair potential of PTH (1–34) combined with 3D printed scaffold-BMSCs on meniscus defect. Thus, this study may provide a reference approach for meniscus tissue engineering in large animals and humans.

**Methods**

**Fabrication of 3D printed Scaffolds**

A native medial meniscus of right knee was acquired from a skeletally mature dog after approval from the Institutional Animal Care and Use Committee of Northwest A & F University. The meniscus was laser scanned (Handy SCAN 700, Creaform, Canada) and the data were imported into process software (Vxelements, Creaform, Canada) to reconstruct three-dimensional (3D) model. The print parameters of a 3D bioprinter (BIOPLATFORM, Medprint, China) were adjusted (Table 1) as described in a previous study[6], and poly(e-caprolactone) (PCL) (Changchun SinoBiomaterials, China) wire (43–50 kDa) was melted and extruded through a heated metal nozzle to print the scaffold.

Simultaneously, the same printing parameters were used to print cylindrical scaffolds, with 5 mm diameter and 3 mm thickness, for the mechanical and cell compatibility tests of the scaffold.

**Isolation and culture of BMSCs**

BMSCs were isolated from canine new stillbirths' (fetus that died during delivery) bone marrows and identified as reported in previous work[24]. BMSCs culture medium consisted of Minimum Essential Medium alpha (α-MEM, Gibco, Billings, MT, USA) supplemented with 10% Fetal Bovine Serum (FBS; Sigma, USA) and 1% penicillin–streptomycin (Sigma).

**Scaffold characterization**

**Microstructure**
The scaffold was observed by scanning electron microscopy (Nova SEM-450, FEI, USA) after freeze-drying and coating with a 5-nm layer of gold on the surface.

**Degradation rate**

The weight of the dry PCL scaffold was recorded as W1, and it was then soaked in phosphate buffered saline (PBS) at 37 °C and pH 7.4. After 4, 8, and 12 weeks, three scaffolds were removed and dried at 45 °C for 24 hours each time, and the weight was recorded as W2. The in vitro degradation rate was calculated according to the formula: Degradation rate (%) = (W1 - W2) / W1 × 100.

**Cytocompatibility**

CCK-8 cytotoxicity test was used to evaluate the compatibility of PCL scaffolds. A total of 100 µL of 3 × 10^4/mL fourth passage BMSCs was cocultured with PCL cylindrical scaffold. At day 3, 5, and 7 of coculture, 10 µL of CCK-8 solution (ZETA life, USA) was added and incubated for 4 hours at 37 °C. Thereafter, the scaffolds were removed and the optical density (OD) at 450 nm was measured. Adherent cultured BMSCs, used as control, were treated in the same manner, three replicates per group.

**Preparation of tissue-engineered meniscus**

**Cell seeding and in vitro culture of scaffold**

The 3D printed PCL scaffolds were sterilized by UV irradiation and soaked in α-MEM. The fourth passage BMSCs were resuspended to 6 × 10^6/mL, 500 µL of the cell suspension was dripped on the upper surface of the scaffold, and the scaffold was placed in an incubator (37 °C, 5% CO2) for 3 hours to aid cell attachment; it was then turned over and the procedure was repeated two times. The BMSCs-scaffold construct was then cultured in chondrogenic induction medium consisting of high-glucose Dulbecco’s modified Eagle’s medium (DMEM; Gibco), 1% penicillin-streptomycin, 40 ng/mL dexamethasone, 50 µg/mL L-proline, 50 µg/mL ascorbate 2-phosphate, 1 mmol/L insulin-transferrin-selenium (ITS), 1 mmol/L sodium pyruvate(all Sigma), 10 ng/mL transforming growth factor-β3 (TGF-β3; Peprotech, USA), and 10 ng/mL bone morphogenetic protein-2 (BMP-2; Peprotech). Cell growth of BMSCs-scaffold construct was observed by inverted phase contrast microscopy.

**Assessment of ECM accumulation at different differentiation culture time points**

BMSCs-scaffold constructs cultured for 7, 14, 21, and 28 days were digested in 125 µg/mL papain
solution (Sigma) at 55 °C overnight, and then centrifuged at 10,000 g for 10 min; the supernatant was collected for glycosaminoglycan (GAG) and DNA determination. GAG quantification was performed using the Blyscan Glycosaminoglycan Assay (Biocolor, UK); briefly, specimens were complexed with Blyscan dye, the absorbance was measured at 656 nm, and the concentration was calculated using a standard curve. DNA content was determined with PicoGreen kit (Invitrogen, USA); sample and dsDNA standard solution were incubated with the Picogreen dye, and the fluorescence value was detected. Ex/Em = 480 nm/520 nm, the DNA concentration of the sample (ng/mL) was calculated using the standard curve. The GAG/DNA ratio was used to evaluate the accumulation of GAG.

Western blotting was used to determine the collagen type II (Col2) expression in the samples. BMSCs-scaffold constructs were treated by western blotting procedure with labeling of Col2(1:1000; Abcam, UK), and immunoblots were visualized by chemiluminescence using a HRP substrate (Millipore, USA). PCNA was used as a loading control.

Animal model
All animal procedures were approved by the Institutional Animal Care and Use Committee of Northwest A & F University. Sixteen mongrel dogs, aged 2–5 years and weighing 7 ± 1 kg, were randomly divided into 4 groups: PTH + BMSCs-scaffold group, BMSCs-scaffold group, Sham group and Meniscectomy group, with 4 dogs in each group.

After surgery preparations and anesthetizing the animals, a medial parapatellar approach[25] (Fig. 1) was used on the right knee of animal to expose the medial meniscus. The capsula articularis was cut laterally along the proximal edge of the medial meniscus, and the entire medial meniscus was removed. For PTH + BMSCs-scaffold group and BMSCs-scaffold group, the tissue-engineered scaffold was placed in the anatomically correct position and then sutured to the anterior and posterior ligaments and the adjacent synovium using 4 − 0 Polyglycolic Acid suture (Ethicon, Johnson & Johnson Medical B.V.); the joint capsule, subcutaneous tissue, and skin were closed gradually with 3 − 0 suture (Ethicon). For Meniscectomy group, only total resection of meniscus was performed, while for Sham group, sham operation was performed involving exposure of the meniscus followed by closure in layers. The operation sites were isolated with sterilized gauze and splinted for external fixation.
Postoperative analgesia and antibiotic prophylaxis were performed for 5 days. The splint was removed 7 days postoperatively, and the animals were taken for regular walks 2 weeks postoperatively to promote knee rehabilitation.

One week postoperatively, animals in PTH combined group were intra-articularly injected on the right knee joint with 24 µg/kg PTH (1-34) (CHINESE PEPTIDE, China) every 2 days for 3 weeks, while the animals in the other groups were injected with the same dose of normal saline.

**Postoperative observation and knee function score**

After operation, the health and rehabilitation conditions of the animals were observed and recorded. Twelve weeks postoperatively, the knee joint function was evaluated by three observers blinded to the groups based on limp, swelling, stair climbing, squatting, and locking, using a modified Lysholm score [25].

**Anatomic observation**

At week 12 after operation, the animals were euthanized and their knee joints were harvested. The menisci, femur, and tibia cartilages were observed and photographed; cartilages were blindly evaluated according to the International Cartilage Repair Society (ICRS) cartilage lesion classification [26] to assess the chondroprotective effects of implants and PTH.

**Histological evaluation**

The implant specimens were fixed in 4% paraformaldehyde and embedded in paraffin, then sectioned into 5 µm thickness, and stained with hematoxylin and eosin (H&E) for general observation, toluidine blue (TB) for the presence of proteoglycans, and picrosirius red (PR) for the presence of collagen type I (Col1). The specimens of femoral condyle and tibial plateaus were fixed in 4% paraformaldehyde and decalcified in 10% ethylenediaminetetraacetic acid for three weeks. They were then embedded in paraffin, sectioned into 7 µm thickness, and stained with H&E for general observation and were blindly graded according to modified Mankin score [27] to evaluate the damages of joint cartilage.

**Immunohistochemistry**

Immunohistochemical analyses were used to evaluate the expression level of the BMSC chondrogenesis terminal differentiation markers collagen type X (Col10), Col1, and matrix metalloproteinases-13 (MMP13) in the implants, as well as the expression of Col2, cartilage
degradation markers MMP13, and A disintegrin and metalloproteinase with thrombospondin motifs 5 (Adamts5) in the tibial plateau cartilage. The antibodies for these proteins were purchased from Bioss, Beijing, China. The DAB substrate system (Zsbio, China) was used for color development. Integrated optical density (IOD) value and area of positive regions of each magnified image was measured with ImageJ 1.58 software (National Institutes of Health, USA). For semiquantitative analyses, the data were expressed as the average optical density (IOD/area).

Statistical analysis

All statistical data were expressed as mean ± standard deviation. SPSS 22.0 statistical software (IBM, USA) was used for statistical analysis. LSD-t test was performed for comparisons of means between two groups and one-way analysis of variance (ANOVA) was used for comparisons among multiple groups. For all tests, P < 0.05 was considered statistically significant.

Results

Characterization of 3D printed scaffold

Structure

The 3D printed meniscus scaffolds reproduced the native meniscus 3D geometry. SEM images showed that the pores in the scaffold were uniformly distributed and had high degree of interconnectivity (Fig. 2-A).

Degradation rate

The scaffolds showed relatively steady degradation in a period of 12 weeks; the in vitro degradation rates at weeks 4, 8, and 12 were 0.78% ± 0.11%, 1.13% ± 0.05%, and 1.48% ± 0.13%, respectively.

Cell compatibility

In CCK-8 assay, the BMSCs showed an increased proliferative tendency during the culturing period (Fig. 2-C); the OD values of the samples at 3, 5, and 7 days after seeding were 0.204 ± 0.023, 0.388 ± 0.044, and 0.596 ± 0.606, respectively, and that of the control group were 0.214 ± 0.025, 0.422 ± 0.033, and 0.616 ± 0.032, respectively. There was no significant difference between the two groups at each time point.

**ECM accumulation of BMSCs-scaffold constructs at different culture time points** *in vitro*

The microscopy images (Fig. 2-B) showed that BMSCs proliferated rapidly on the scaffold. The GAG/DNA ratio of the BMSCs-scaffold constructs increased with culture time, and reached its peak at
day 21, which was about twice the value at day 7, and then decreased at day 28 (Fig. 2-D). In line with GAG/DNA determination, the expression of Col2 in the BMSCs-scaffold constructs increased continually until day 21 of culture; the gray value analysis showed that the expression on day 21 was significantly higher than that on day 14 and day 28 (P < 0.05, Fig. 2-E). Therefore, the BMSCs-scaffold cultured for 21 days in vitro was selected for in vivo implantation.

Postoperative observations and knee function score
All animals recovered well without infection. Twelve weeks postoperatively, dogs in the Meniscectomy group were capable of walking but found running difficult; those in BMSCs-scaffold group demonstrated normal walking and running, despite a slight limp in two dogs; whereas those in the PTH + BMSCs-scaffold group exhibited normal walking, running, and climbing of stairs, which was the same as that observed in the Sham group. Lysholm scores for knee joint function (Fig. 3-B) of the PTH + BMSCs-scaffold group (36.59 ± 2.04), BMSCs-scaffold group (33.25 ± 2.27), and Sham group (43.00 ± 1.63) were all significantly higher (P < 0.01) than that of the Meniscectomy group (10.67 ± 5.21). Further, the score for the PTH + BMSCs-scaffold group was significantly higher than that of the BMSCs-scaffold group (P < 0.05).

Anatomic observations
The anatomic observation (Fig. 3-A) found that new tissue formed in all the implants, and most of the scaffolds maintained the original shape and size without fracture or fragmentation. Compared to the BMSCs-scaffold group, PTH + BMSCs-scaffold group formed more neocartilage-like tissue on the surface of the implant, which was smoother and better integrated with the surrounding tissue.

In meniscectomy group, the volume of joint fluid increased in the joint cavity, connective tissue was newly formed, and the joint cartilage surface was severely abraded. For BMSCs-scaffold group, both the femoral condyle and tibial plateau surface were abraded to some extent. In the PTH treated group, there was almost no abrasion on the femoral condyle surface and slight abrasion on the tibial plateau surface, which was similar to those in Sham group. According to the ICRS cartilage lesion classification (Fig. 3-C), the score for femur cartilage in BMSCs-scaffold group (0.92 ± 0.17) was significantly lower than that in Meniscectomy group (2.42 ± 0.32) (P < 0.01), but significantly higher
than that in Sham group (0.17 ± 0.19); while PTH + BMSCs–scaffold group score (0.42 ± 0.17) was significantly lower than that in BMSCs–scaffold group (P < 0.01), and slightly higher than that in Sham group with no significant difference. Similar to femoral condylar cartilage, the tibial plateau cartilage defect scores for the PTH + BMSCs–scaffold group (0.42 ± 0.17) and BMSCs–scaffold group (1.24 ± 0.17) were significantly lower than that for the Meniscectomy group (2.75 ± 0.17). PTH + BMSCs–scaffold group score showed no significant difference as compared to that of the Sham group (0.17 ± 0.19), but was significantly lower than that of the BMSCs–scaffold group (P < 0.01).

**Histological evaluation of implants**

H&E staining showed that (Fig. 4-A), the implanted scaffolds were filled with new tissue; however, there were more voids and connective tissue observed in the new tissue of BMSCs–scaffold group, while the new tissue in PTH + BMSCs–scaffold group was more uniform. In the enlarged image of H&E staining (Fig. 4-B), PTH treated group showed a large number of round-shaped chondrocyte-like cells in the intermediate and inner regions of the implants, which were embedded in lacuna structure, and vascularization and spindle-shaped fibroblast-like cell distribution in the outer regions; these observations were similar to those in the native meniscus of the Sham group. For BMSCs–scaffold group, there were more spindle-shaped fibroblast-like cells and less chondrocyte-like cells, with no cartilage islands in the intermediate regions. In case of TB staining and PR staining (Fig. 4-B), PTH treated group showed stronger staining than BMSCs–scaffold group. Further the staining distribution in this group was similar to that seen in the native meniscus, indicating the deposition of more cartilage ECM components proteoglycans and Col1 in the neo-tissue of implants in PTH + BMSCs–scaffold group.

**Immunohistochemical evaluation of implants**

According to the immunohistochemical staining image (Fig. 5-A), terminal differentiation markers Col10 and MMP13 were markedly observed in the cytoplasm in intermediate and inner regions of the implants in BMSCs–scaffold group as compared with Sham group, while they were observed only in the inner region of implants in PTH + BMSCs–scaffold group. The IOD/area value of immunohistochemistry images (Fig. 5-B) supported these observations. The IOD/area value for Col10
in BMSCs–scaffold group (0.28 ± 0.02) was significantly higher than that in Sham group (0.18 ± 0.01) (P < 0.01), and significantly lower in PTH + BMSCs–scaffold group (0.20 ± 0.01) (P < 0.01). The IOD/area value for MMP13 in PTH + BMSCs–scaffold group (0.39 ± 0.01) and BMSCs–scaffold group (0.48 ± 0.02) were significantly higher than that in the Sham group (0.31 ± 0.01) (P < 0.01); however, the value in PTH + BMSCs–scaffold group was significantly lower than that in BMSCs–scaffold group (P < 0.01).

Histology and immunohistochemical evaluation of knee articular cartilage

In the HE staining (Fig. 6-A), both femur and tibia cartilage showed clefts and hypo-cellularity in Meniscectomy group, and some of the clefts of the tibia cartilage progressed to the radial zone. In BMSCs–scaffold group, cartilage damage was reduced but still showed irregular clefts to the transitional zone and hypo-cellularity in the superficial zone. In PTH + BMSCs–scaffold group, the degenerative changes of cartilage were less severe, irregular clefts and hypo-cellularity were observed only in the surface of tibia cartilage, and the femur cartilage were well-preserved. According to the Mankin score (Fig. 6-B), the femur cartilage damage degree in the BMSCs–scaffold group (4.50 ± 1.23) was significantly lower than that in Meniscectomy group (8.92 ± 0.96), but higher than that in Sham group (0.33 ± 0.27) (P < 0.01), and significantly decreased with PTH combination (2.33 ± 0.72) (P < 0.05). Mankin score for the tibia cartilage in BMSCs-scaffold group (5.33 ± 0.72) was significantly lower than that in Meniscectomy group (10.08 ± 1.00) and higher than that in Sham group (0.17 ± 0.19) (P < 0.01), and the score in PTH + BMSCs–scaffold group (3.25 ± 1.40) was significantly lower than that in BMSCs–scaffold group (P < 0.05).

The immunohistochemistry analysis (Fig. 6-C) found decreased staining of cartilage matrix Col2 and increased staining of matrix degeneration markers Adamts5 and MMP13 in all the other three groups when compared with Sham group. The corresponding IOD/area value (Fig. 6-D) showed that BMSCs–scaffold (0.31 ± 0.02) significantly impeded Col2 reduction of meniscectomy (0.28 ± 0.03) (p < 0.01), and the combination of PTH (0.33 ± 0.01) reserved more Col2 (p < 0.05). For Adamts5, the IOD/area values were as follows: Meniscectomy group (0.59 ± 0.07) > BMSCs–scaffold group (0.54 ± 0.03) > PTH + BMSCs–scaffold group (0.46 ± 0.03) > Sham group (0.41 ± 0.03), where the significant
difference was observed across all four groups (p < 0.01). The IOD/area difference of MMP13 was similar to that of Adamts5: Meniscectomy group (0.60 ± 0.03) > BMSCs-scaffold group (0.56 ± 0.01) > PTH + BMSCs-scaffold group (0.52 ± 0.03) > Sham group (0.43 ± 0.19), and the difference was significant across all four groups, respectively (p < 0.01).

Discussion
The purpose of this study was to optimize the 3D printed PCL meniscus scaffolds seeded with BMSCs and cultured in vitro, and to investigate the effect of PTH (1–34) on the repairing of the tissue-engineered meniscus in vivo after implantation in total meniscectomy canine model. The results showed that the BMSCs-scaffold construct synthesized and accumulated more cartilage ECM in vitro when cultured for 21 days as compared to other culture time points. Furthermore, intra-articular injection of PTH (1–34) decreased the cell hypertrophy of the tissue-engineered meniscus during regeneration and increased the chondroprotective effects of the tissue-engineered meniscus for knee cartilage in vivo. This demonstrated the satisfactory efficacy of PTH (1–34) and tissue-engineered meniscus combination on meniscal replacement.

There are various methods to prepare tissue-engineered meniscus scaffolds. However, 3D printing provides a high controllability of the internal structure and geometric shape and personalization of printed entities[5]. Due to the complicated anatomical structure and stress environment of meniscus, 3D printing has greater advantages in the preparation of tissue-engineered meniscus scaffold as compared to other fabrication technologies[26]. In this study, a PCL scaffold was 3D printed to prepare tissue-engineered meniscus scaffolds, which showed reproduction of the native meniscus anatomical shape, low degradation rate, and good cell compatibility, and could anatomically respond to the knee joint pressure as well as support the growth of cells and tissues in vivo.

Currently, there is no uniform standard for in vitro culture of cell-seeded scaffolds in tissue-engineered meniscus studies. In many studies, cell-free scaffold or cell-seeded scaffold were transplanted without in vitro culture into animals; although certain regeneration effects were achieved, joint degeneration often occurred after transplantation[27, 28]. In this study, 3D printed scaffolds were seeded with canine BMSCs and cultured under chondrogenic culture medium in vitro,
and the BMSCs–scaffold showed an increasing trend of cell proliferation and ECM, GAG, and Col2 synthesis, which reached the highest levels on day 21 of culture, and declined on day 28; the decline may be related to hypertrophy of BMSCs during long-term chondrogenic induction in vitro[12]. Therefore, the BMSCs–scaffold construct was cultured in vitro for 21 days before implantation, which makes it more conducive to the synthesis and accumulation of cartilage ECM, resulting in more mature structure and function, so that it responds more quickly to knee joint pressure after implantation.

BMSCs are frequently used as seed cells for tissue-engineered meniscus[10, 29]; however, chondrogenesis leads to undesired terminal differentiation of the generated chondrocyte[30], which reduces the quality of regenerative tissue and decreases its repair efficacy [31]. In our study, the immunohistochemistry of meniscus implants in BMSCs–scaffold group showed high expression of the chondrocyte terminal differentiation markers Col10 and MMP13, indicating the terminal differentiation of BMSCs. PTH (1–34) has demonstrated its potential in inhibiting the terminal differentiation of BMSC chondrogenesis[16], and it is often used in research concerning cartilage tissue engineering[20].

Mueller et al. [17] found that intermittent PTHrP stimulation can promote cartilage formation and inhibit hypertrophy of BMSC chondrogenesis in vitro. Among the four PTHrP isoforms (1–34, 1–86, 7–34, and 107–139), Lee et al.[32] found that PTHrP (1–34) most significantly enhanced chondrogenesis and inhibited hypertrophic differentiation of human BMSCs. In this study, at 12 weeks postoperatively, the cells composition and ECM deposition of the neo-tissue of meniscus implants in PTH treated group were similar to those of native meniscus. Furthermore, this tissue also showed lower expression of terminal differentiation markers Col10 and MMP13. These observations suggested that intra-articular injection of PTH (1–34) enhanced tissue regeneration and ECM deposition as well as inhibited the terminal differentiation of tissue-engineered meniscus with BMSCs as seed cells in vivo.

At the same time, we also observed the chondroprotective effect of PTH (1–34) on the tissue-engineered meniscus implant. Although recent studies have attempted to make tissue-engineered meniscus that simulate the anatomy and mechanical properties of native meniscus in order to
alleviate the stress environment of knee joint and better protect the knee cartilage, their protective
effect after transplantation needs to be strengthened[3, 33, 34]. Hannink et al.[14] implanted PCL-PU
meniscus scaffolds in dogs and found articular cartilage degeneration and chondrocyte hypertrophy.
Similar to their findings we also found that the articular cartilage in the tissue of BMSCs–scaffold
group showed significant damage, although it showed a certain reduction in cartilage degeneration
and chondrocyte loss when compared with the Meniscectomy group. This may be due to the variation
of cartilage friction coefficient in the knee joint after the transplantation, resulting in wear on the
cartilage surface, meanwhile, the biomechanical properties of knee joint may have changed, which
would disrupt the normal homeostasis of the joint, leading to cartilage degeneration[34–36]. The
degeneration of cartilage may also have an impact on the meniscus function[37]. Adamts5 and
MMP13 are considered important catabolic enzymes that degrade aggrecan(AGG) and Col2, key ECM
components of functional cartilage, and their expression is related to the cartilage degeneration[19,
38, 39]. The potential of PTH (1–34) in protecting against cartilage degeneration and inducing matrix
regeneration after articular cartilage injury has been demonstrated in in vivo studies[19, 32, 40]. Dai
et al. subcutaneously injected PTH (1–34) into guinea pigs of meniscectomy model and found the
inhibition of cartilage degeneration by PTH (1–34), which may be related to the inhibition of Adamts4
and MMP13 expression[41]. In this study, the injection of PTH (1–34) after transplantation of tissue-
engineered meniscus reduced the degree of lesions in the knee cartilage showed a higher expression
of Col2 and lower expression of Adamts5 and MMP13. This suggested that PTH (1–34) inhibited the
degeneration of cartilage caused by the total substitution of tissue-engineered meniscus, protected
the integrity of the knee joint cartilage, and thus enhanced the repairing effects of tissue-engineered
meniscus.
There are several limitations of this study. The recent studies report different application dosage,
frequency, and duration of PTH (1–34)[18, 42, 43]. The regeneration of tissue-engineered meniscus in vivo is a multi-step dynamic process, and the variations in PTH (1–34) application to a certain factor
may cause different effects[21, 44]. In the present study, although the PTH (1–34) promoted the
effects of BMSCs-3D printed scaffold for total meniscal substitution, cartilage degradation was not
completely prevented. Therefore, further optimization of the administration time, dosage, and mode is needed, and further follow-up studies should be undertaken to enhance the adjuvant effect of PTH (1–34) on the regeneration of tissue-engineered meniscus with BMSCs as seed cells in vivo.

Conclusions
In this study, we found that 21 days is the optimal time for in vitro culturing of the tissue-engineered meniscus based on 3D-printed PCL scaffold seeded with canine BMSCs. Further, PTH (1–34) application promoted the regenerative and chondroprotective effects of the tissue-engineered meniscus total implantation in a canine model by inhibiting the terminal differentiation of BMSCs chondrogenesis and degeneration of knee joint cartilage. Thus, this therapeutic combination represents a promising method to increase the chance of regeneration in tissue-engineered meniscus.

Abbreviations
BMSCs
Bone marrow mesenchymal stem cells
3D
Three-dimensional
PTH
Parathyroid hormone
PTHrP
Parathyroid hormone related protein
PCL
Poly(e-caprolactone)
DMEM
Dulbecco’s modified Eagle’s medium
GAG
Glycosaminoglycan
ECM
Extracellular matrix
OD value
Optical density value
ICRS
International Cartilage Repair Society
H&E staining
Hematoxylin and eosin staining
TB staining
Toluidine blue staining
PR staining
Picrosirius red staining
COL
Collagen
MMP13
Metalloproteinases-13
Adams5
A disintegrin and metalloproteinase with thrombospondin motifs 5

Declarations

Ethics approval and consent to participate
All animal experiments in this study were approved by the Animal Ethics Committee of Northwest A&F University.

Consent for publication
All co-authors gave consent for publication.

Availability of data and material
All data have been included in this article and its supplementary information files.

Competing interests
The authors indicate no potential conflicts of interest.

Funding
This study was supported by National Natural Science Foundation of China (No. 31572577).

Authors’ contributions
Wen Zhao and Tong Zou carried out the experiments, analysed the data and drafted the manuscript.
Yihua Zhang designed the study, reviewed data and revised the manuscript. Hao Cui, Yangou Lv, Dengke Gao, Chenmei Ruan and Xia Zhang conducted parts experiments and helped with the surgery. All authors read and approved the final manuscript.

Acknowledgements
The authors would like to thank the Experimental Animal Center of Northwest A&F University for providing test animal, the Animal Ethics Committee of Northwest A&F University for proving fund.

References
1. Sanchezadams J, Athanasiou KA. The Knee Meniscus: A Complex Tissue of Diverse Cells. Cellular Molecular Bioengineering. 2009;2(3):332-40.
2. Advanced Regenerative Strategies for Human Knee Meniscus
   Cengiz IF, Silva-Correia J, Pereira H, Espregueira-Mendes J, Oliveira JM, Rui LR.
   Advanced Regenerative Strategies for Human Knee Meniscus 2017.
3. Gao S, Chen M, Wang P, Li Y, Yuan Z, Guo W, et al. An electrospinning fiber reinforced scaffold promoted total meniscus regeneration in rabbit meniscectomy model. Acta Biomater. 2018;73:1742706118302022.
4. Bilgen B, Jayasuriya CT, Owens BD. Current Concepts in Meniscus Tissue Engineering and Repair. Advanced Healthcare Materials. 2018;7(11):1701407.
5. Zhang ZZ, Wang SJ, Zhang JY, Jiang WB, Huang AB, Qi YS, et al. 3D-Printed Poly(ε-caprolactone) Scaffold Augmented With Mesenchymal Stem Cells for Total Meniscal Substitution. Am J Sports Med. 2017;45(5):363546517691513.
6. Szojka A, Lahl K, Andrews SHJ, Jomha NM, Osswald M, Adesida AB. Biomimetic 3D printed scaffolds for meniscus tissue engineering. Bioprinting. 2017;8:1-7.
7. Lee CH, Rodeo SA, Fortier LA, Lu C, Erisken C, Mao JJ. Protein-releasing polymeric scaffolds induce fibrochondrocytic differentiation of endogenous cells for knee meniscus regeneration in sheep. Science Translational Medicine. 2014;6(266).
8. Murphy CA, Costa JB, Silvacorreia J, Oliveira JM, Reis RL, Collins MN. Biopolymers and Polymers in the Search of Alternative Treatments for Meniscal Regeneration: State of the Art and Future Trends. Applied Materials Today. 2018;12(2018):51-71.
9. Zellner J, Mueller MB, Berner A, Dienstknecht T, Kujat R, Nerlich M, et al. Role of
mesenchymal stem cells in tissue engineering of meniscus. Journal of Biomedical Materials Research Part A. 2010;94(4):1150-61.

10. Niu W, Guo W, Han S, Zhu Y, Liu S, Guo Q. Cell-Based Strategies for Meniscus Tissue Engineering. Stem Cells International. 2016;2016:4717184-.

11. Makris EA, Gomoll AH, Malizos KN, Hu JC, Athanasiou KA. Repair and tissue engineering techniques for articular cartilage. Nat Rev Rheumatol. 2015;11(1):21-34.

12. Fischer J., Dickhut A,., Rickert M, Richter. W,. Human articular chondrocytes secrete parathyroid hormone-related protein and inhibit hypertrophy of mesenchymal stem cells in coculture during chondrogenesis. Arthr Rhuem. 2014;62(9):2696-706.

13. Weiss S,, Hennig T, Bock,. R, Steck E, Richter. W,. Impact of growth factors and PTHrP on early and late chondrogenic differentiation of human mesenchymal stem cells. J Cell Physiol. 2010;223(1):84-93.

14. Hannink G, van Tienen TG, Schouten AJ, Buma P. Changes in articular cartilage after meniscectomy and meniscus replacement using a biodegradable porous polymer implant. Knee Surg Sports Traumatol Arthrosc. 2011;19(3):441-51.

15. Warnecke D, Stein S, Haffner-Luntzer M, Roy LD, Skaer N, Walker R, et al. Biomechanical, Structural and Biological Characterisation of a New Silk Fibroin Scaffold for Meniscal Repair. Journal of the Mechanical Behavior of Biomedical Materials. 2018:S175161618303928-.

16. Fischer J, Ortel M, Hagmann S, Hoeflich A, Richter W. Role of PTHrP(1-34) Pulse Frequency Versus Pulse Duration to Enhance Mesenchymal Stromal Cell Chondrogenesis. Journal of Cellular Physiology. 2016;231(12):n/a-n/a.

17. Mueller MB, Maria F, Johannes Z, Arne B, Thomas D, Richard K, et al. Effect of parathyroid hormone-related protein in an in vitro hypertrophy model for mesenchymal stem cell chondrogenesis. Int Orthop. 2013;37(5):945-51.
18. Je-Ken C, Ling-Hwa C, Shao-Hung H, Shun-Cheng W, Hsin-Yi L, Yi-Shan L, et al. Parathyroid hormone 1–34 inhibits terminal differentiation of human articular chondrocytes and osteoarthritis progression in rats. Arthr Rhuem. 2014;60(10):3049–60.

19. Chen S, Fu P, Cong R, Wu H, Pei M. Strategies to minimize hypertrophy in cartilage engineering and regeneration. Genes Diseases. 2015;2(1):76-95.

20. Chen Y, Chen Y, Zhang S, Du X, Bai B. Parathyroid Hormone-Induced Bone Marrow Mesenchymal Stem Cell Chondrogenic Differentiation and its Repair of Articular Cartilage Injury in Rabbits. Medical Science Monitor Basic Research. 2016;22:132-45.

21. Sampson ER, Hilton MJ, Tian Y, Chen D, Schwarz EM, Mooney RA, et al. Teriparatide as a Chondroregenerative Therapy for Injury-Induced Osteoarthritis. 2011;19(101):S227-S.

22. Orth P, Cucchiarini M, Zurakowski D, Menger MD, Kohn DM, Madry H. Parathyroid hormone [1–34] improves articular cartilage surface architecture and integration and subchondral bone reconstitution in osteochondral defects invivo. Osteoarthritis Cartilage. 2013;21(4):614-24.

23. Zhang W, Chen J, Tao J, Hu C, Chen L, Zhao H, et al. The promotion of osteochondral repair by combined intra-articular injection of parathyroid hormone-related protein and implantation of a bi-layer collagen-silk scaffold. Biomaterials. 2013;34(25):6046-57.

24. Zhang Y. Transplantation of amniotic scaffold seeded mesenchymal stem cells and/or endothelial progenitor cells from bone marrow to efficiently repair 3-cm circumferential urethral defect in model dogs. Tissue Engineering Part A.ten.TEA.2016.0518.

25. Lee H, Lee SY, Na YG, Kim SK, Yi JH, Lim JK, et al. Surgical Techniques and
Radiological Findings of Meniscus Allograft Transplantation. European Journal of Radiology. S0720048 x 16301462.

26. Zhang Z, Jiang D, Ding J, Wang S, Zhang L, Zhang J, et al. Role of scaffold mean pore size in meniscus regeneration. Acta Biomater. 2016;43:314–26.

27. Li P, Feng X, Jia X, Fan Y. Influences of tensile load on in vitro degradation of an electrospun poly(l-lactide-co-glycolide) scaffold. 6(8):2991–6.

28. Moradi L, Vasei M, Dehghan MM, Majidi M, Farzad Mohajeri S, Bonakdar S. Regeneration of meniscus tissue using adipose mesenchymal stem cells-chondrocytes co-culture on a hybrid scaffold: In?vivo study. Biomaterials. 126:18–30.

29. Chew E, Prakash R, Khan W, Chew E, Prakash R, Khan W, et al. Mesenchymal stem cells in human meniscal regeneration: A systematic review. 2017;24(C):3–7.

30. Bomer N, Hollander WD, Suchiman H, Houtman E, Slieker RC, Heijmans BT, et al. Neo-cartilage engineered from primary chondrocytes is epigenetically similar to autologous cartilage, in contrast to using mesenchymal stem cells. Osteoarthritis Cartilage. 2016;24(8):1423–30.

31. Wei Z, Chen J, Zhang S, Hong WO. Inhibitory function of parathyroid hormone-related protein on chondrocyte hypertrophy: the implication for articular cartilage repair. Arthritis Research Therapy. 2012;14(4):221-.

32. Lee JM, Im GI. PTHrP isoforms have differing effect on chondrogenic differentiation and hypertrophy of mesenchymal stem cells. Biochemical Biophysical Research Communications. 2012;421(4):819–24.

33. Rongen JJ, Tienen TGV, Bochove BV, Grijpma DW, Buma P. Biomaterials in search of a meniscus substitute. Biomaterials. 2014;35(11):3527–40.

34. Rongen JJ, Hannink G, Van Tienen TG, et al. The protective effect of meniscus allograft transplantation on articular cartilage: a systematic review of animal studies.
Osteoarthritis & Cartilage. 23(8):1242–53.

35. Owens BD, Tabaddor RR. Meniscus Injuries. Clin Sports Med. 2020;39(1):i.

36. Yuan XL, Meng HY, Wang YC, Peng J, Guo QY, Wang AY, et al. Bone-cartilage interface crosstalk in osteoarthritis: potential pathways and future therapeutic strategies. Osteoarthritis Cartilage. 2014;22(8):1077–89.

37. Fischenich KM, Lewis JT, Kindsfater KA, et al. Effects of degeneration on the compressive and tensile properties of human meniscus. Journal of Biomechanics. 48(8):1407–11.

38. Tchetina EV, Squires G, Poole AR. Increased type II collagen degradation and very early focal cartilage degeneration is associated with upregulation of chondrocyte differentiation related genes in early human articular cartilage lesions. 2005;32(5):876–86.

39. Miriam B, Laura L, Roman-Blas JA, Santos CE, Caeiro JR, Sonia D, et al. Subchondral bone microstructural damage by increased remodelling aggravates experimental osteoarthritis preceded by osteoporosis. Arthritis Research Therapy. 2010;12(4):R152. ,12,4(2010-08-02).

40. Morita Y, Ito H, Ishikawa M, Fujii T, Furu M, Azukizawa M, et al. Subchondral bone fragility with meniscal tear accelerates and parathyroid hormone decelerates articular cartilage degeneration in rat osteoarthritis model. Journal of Orthopaedic Research.

41. Dai MW, Chu JG, Tian FM, Song HP, Wang Y, Zhang YZ, et al. Parathyroid hormone(1-34) exhibits more comprehensive effects than celecoxib in cartilage metabolism and maintaining subchondral bone micro-architecture in meniscectomized guinea pigs. Osteoarthritis Cartilage. 2016;24(6):1103–12.

42. Kudo S, Mizuta H, Takagi K, Hiraki Y. Cartilaginous repair of full-thickness articular
cartilage defects is induced by the intermittent activation of PTH/PTHrP signaling. 19(7):886-94.

43. Chang LH, Wu S-C, Chen C-H, Wang G-J, Chang J-k, Ho M-L. Parathyroid hormone 1–34 reduces dexamethasone-induced terminal differentiation in human articular chondrocytes. Toxicology. 368-369:116 – 28.

44. Dhillon RS, Schwarz EM. Teriparatide Therapy as an Adjuvant for Tissue Engineering and Integration of Biomaterials. Materials. 4(12):1117–31.

Tables

Table 1  Print parameters of meniscus scaffold

| Print Property        | Parameters          |
|-----------------------|---------------------|
| PCL wire diameter     | 1.75 mm             |
| Temperature set point | 90 °C               |
| Fiber Diameter        | 0.3 mm              |
| Layer thickness       | 0.3 mm              |
| Wall thickness        | 0.8 mm              |
| Porosity              | 60%                 |
| Pore interconnectivity| 100%                |
| Mass ($\pm$ SD, n = 10)| 0.0894 ± 0.0084 g   |

Figures
Figure 1

Implantation process of tissue-engineered meniscus (A) Cut the skin, fascia, and joint capsule; (B) Separating medial meniscus; (C) Resection of medial meniscus; (D) Transplanted tissue-engineered and sutured meniscal implant; (E) Suture joint capsule; (F) Sutured skin incision.
Preparation of tissue-engineered meniscus. (A) Characteristics of 3D printed scaffold. (a) canine native meniscus, (b) reconstruction model of canine native meniscus, (c) general observation of 3D printed meniscus scaffold, (d–f) scanning electron microscope images of 3D printed PCL scaffold; (B) Inverted phase contrast microscope image of BMSCs-scaffold constructs cultured for 7 d and 14 d; (C) CCK-8 assay for determination of the cell compatibility of 3D printed PCL scaffold; (D) GAG/ DNA ratios of BMSC-scaffold constructs at different culture time points; (E) The relative expression levels of Col2 in BMSCs-scaffold constructs at different culture time points (* P < 0.05, ** P < 0.01).
Figure 3

Anatomic observation and scores of knee joint. (A) Anatomic observation of knee cartilage and meniscus implant (arrows indicate the cartilage injury). (B) Lysholm score, PTH + BMSCs-scaffold group presented better knee joint function than BMSCs-scaffold group. (C) ICRS score, the cartilage defect degree of femoral condyle and tibial plateau extent in PTH + BMSCs-scaffold group was less than that in BMSCs-scaffold group, and showed no significant difference with that in the Sham group (* P < 0.05, ** P < 0.01)
Figure 4

Histological observation of meniscus implants. (A) H&E staining. The general view on the left (scale bar = 100 μm) shows the structure of outer, intermediate, and inner regions of the new tissue of the meniscus implants, and the high-magnification images on the right (scale bar = 25 μm) correspond to the images inserted in the frame in the left view, respectively. A large number of chondrocyte-like cells (black arrows) were observed in the intermediate and inner regions in PTH + BMSCs-scaffold group implants, which were similar to the native meniscus in the Sham group, while mostly fibroblast-like cells (triangles) were observed in the intermediate region of the implants of BMSCs-scaffold group. (B) Toluidine blue (TB) and Picrosirius Red (PR) staining showed the deposition of proteoglycan and collagen type I in
the new tissue of meniscus implants and native meniscus, respectively. PTH + BMSCs-scaffold group had a stronger staining than BMSCs-scaffold group (Scale bar = 100 μm). * marked the dissolution position of the PCL scaffold in the process of sectioning.

Figure 5

(A) Immunohistochemical staining for Col10 and MMP13 of meniscus implants. (Scale bar = 100 μm). (B) Immunohistochemical analyses of Col10 and MMP13 of meniscus implants. Values for integrated optical density per area (IOD/area) of Col10 and MMP13 were lower in PTH + BMSCs-scaffold group compared with that in BMSCs-scaffold group (* P < 0.05, ** P < 0.01).
Figure 6

Histological and immunohistochemical observation and evaluation of the knee joint
cartilage. (A) H&E staining of the femoral condyle and tibial plateau cartilage (scale bar = 250 μm). (B) Mankin score of histology of femoral condyle and tibial plateau cartilage. PTH + BMSCs-scaffold group showed lower cartilage degeneration than the BMSCs-scaffold and the Meniscectomy groups. (C) Immunohistochemical staining of tibial plateau for cartilage ECM component Col2 and matrix degradation markers Adamts5 and MMP13 (scale bar = 100 μm). (D) Semi-quantitative analysis of immunohistochemical staining. Values for integrated optical density per area (IOD/area) of Col2 in PTH + BMSCs-scaffold group were higher and those for Col10 and MMP13 were significantly lower than that in the BMSCs-scaffold group. (* P < 0.05, ** P < 0.01).