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EXPERIMENTAL STUDY

Effect of Ermiao Fang with Xixin (*Herba Asari Mandshurici*) on bone marrow stem cell directional homing to a focal zone in an osteoarthritis rat model

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Abstract

OBJECTIVE: To investigate the effects of Ermiao Fang (EM) with medical guide Xixin (*Herba Asari Mandshurici*) (HAM) on bone marrow stem cell migration to a focal zone in osteoarthritis (OA) rats.

METHODS: OA rats were induced by arthrectomy and assigned to sham-operated, model, EM, or EM plus HAM groups. All rats were injected with recombinant human granulocyte colony-stimulating factor 30 $\mu g \cdot kg^{-1} \cdot d^{-1}$ for 7 days and treated with EM or EM plus HAM at 1.6 or 1.9 $g \cdot kg^{-1} \cdot d^{-1}$ for 3 or 6 weeks, respectively. Chondrocyte apoptosis and cartilage matrix components were tested by transferase-mediated deoxyuridine triphosphate-biotin nick end labeling assay and special staining. Levels of interleukin-1 beta (IL-1β) tumor necrosis factor alpha (TNF-α) nitric oxide (NO), and inducible nitric oxide synthase (iNOS) in serum were detected by enzyme-linked immunosorbent assay or radioimmunoassay. Matrix metalloproteinases (MMPs)-13, tissue inhibitors of metalloproteinases (TIMPs)-1, Bromodeoxyuridine (BrdU), cluster of differentiation 34 (CD34), and stromal cell-derived factor 1 (SDF-1) were measured by immunohistochemical assay.

RESULTS: The EM and EM plus HAM groups had significantly less cartilage damage and synovium inflammation the model group. Moreover, the EM and EM plus HAM groups had less chondrocyte apoptosis and more proteoglycan and collagen content than the model group. The EM and EM plus HAM groups had obviously higher MMPs-13 and TIMPs-1 expression in the cartilage than the model group. Moreover, the two formula groups had less release of IL-1β, TNF-α, NO, and iNOS than model group. Importantly, the expressions of BrdU, CD34, and SDF-1 in cartilage were significantly higher in the EM and EM plus HAM-Medtreated rats than model group. Notably, the EM plus HAM treatment seemed to have the greatest effects.

CONCLUSION: HAM improves the therapeutic effects of EM on OA rats by enhancing BMSC directional homing to the focal zone.

Key words: Osteoarthritis; Myeloid progenitor cells; Homing; Ermiao Fang; Xixin (*Herba Asari Mandshurici*)

INTRODUCTION

Osteoarthritis (OA) is a degenerative joint disease characterized by chondrocyte function loss and extracellular matrix destruction.[1] Surgical and pharmaceutical interventions are difficult for restoration of normal cartilage function.[2] Bone marrow stromal cells (BMSCs) have chondrocyte differentiated potency[3] and are induced with transforming growth factor beta3 (TGF-β 3) to stimulate proteoglycan and collagen type Ⅱ synthesis.[4,5] This synthesis appears to be ideal in therapeutic use for cartilage regeneration and is a new field of study in the application of stem cells. BMSCs can be summoned by inflammatory irritation of lesions. However, the effect of BMSC homing to a focal zone is limited.[6] Therefore, devising an agent or method that can promote BMSC homing to pathologic tissues is important.

Ermiao Fang (EM) is a compound prescription composed of Huangbai (*Cortex Phellodendri Amurensis*) and Cangzhu (*Rhizoma Atractylodis Lanceae*) in a specified ratio.[7] EM has been used as treatment for arthralgia syndrome (Bi syndrome) in Traditional Chinese Medicine since the Ming Dynasty to clear heat and remove dampness. Today in clinic, EM is also a basic prescription for treating OA.[8] Moreover, various medical guide herbs lead to better curative effects. For example, Xixin (*Herba Asari Mandshurici*) (HAM), as a medical guide, is used in the treatment for arthralgia.[9] Although medicinal guide has an obvious effect-directing function, its link to the application of medicinal guide and homing of BMSCs has been uncharacterized. This study is designed to employ granulocyte colony-stimulating factor (G-CSF) to mobilize BMSCs to peripheral blood and then observe the influence of EM formula plus HAM, a medical guide, on mobilized BMSC homing to an osteoarthritic damaged area.

MATERIALS AND METHODS

Chemicals and reagents

Matrix metalloproteinase (MMP)-13 antibody (275628) and tissue inhibitors of metalloproteinase (TIMP)-1 antibody were purchased from Abcam (Cambridge, UK). Tunel kit (08c11b), Brdu antibody (bu330201), CD34 antibody (10cm61), stromal cell derived factor (SDF) -1 antibody (Y-07c29c), and type II collagen antibody (10 cm222) were purchased from Boster (Wuhan, China). All other chemicals were of analytical grade.

Herbal preparation

EM was prepared as described in the Chinese Pharmacopeia of 2010.[7] Briefly, the three ingredients, Cangzhu (*Rhizoma Atractylodis Lanceae*) (500 g, fried with salt) and Huangbai (*Cortex Phellodendri Amurensis*) 500 g, and Xixin (*Herba Asari Mandshurici*) (HAM, 200 g) were pulverized to fine powder, suspended in distilled water to a concentration of 0.16 g/mL (EM) and 0.19 g/mL (EM + HAM), respectively, and mixed well before administration.

OA animal model and treatment

Forty-eight male Sprague-Dawley rats of clean grade, 4-months-old, weighing (200-220) g, were purchased from Experimental Animal Center of Academy Of Military Medical Sciences (Certificate No. SCXK [jun] 2007-004) and were anesthetized with halothane. After being shaved and disinfected, the right knee joint was exposed with a medial par patellar approach. The patella was dislocated laterally and the knee placed in full flexion, followed by anterior cruciate ligament transection and medial meniscus resection (ACLT + MMx) with micro-scissors. Sham-arthrotomized animals were negative controls. The rats were randomly divided into four groups with a random number table method: ACLT+MMx without treatment (Model, n=12), sham operated (Sham, n=12), ACLT+MMx rats treated with EM intragastrically at a daily dose of 1.6 g/kg (EM, n= 12), and treated with EM (1.6 g/kg) plus HAM (0.3 g/kg) at a daily dose of 1.9 g/kg for 3 or 6 weeks (EM plus HAM, n=12). Dose calculations followed guidelines correlating dose equivalents between humans and laboratory animals, on the basis of ratios of body surface area.[10] The dose equivalents 1.6 or 1.9 g/kg are based on recommended doses of 0.25 or 0.3 g/kg in humans, respectively. Untreated control rats induced by ACLT+MMx and sham rats received distilled water only. Meanwhile, all animals were subcutaneously injected with recombinant human G-CSF (rhG-CSF) at a dose of 30 $\mu g \cdot kg^{-1} \cdot d^{-1}$ for 7 days after arthrectomy. To label BMSCs, all rats were intraperitoneally injected with BrdU (50 mg/kg) for 3 days before sampling at the end of the 3rd and 6th week. All procedures for consideration of animal welfare were reviewed and approved by the ethical committee of China Academy of Traditional Chinese Medicine.

Count of peripheral white blood cells

Before (0 day) and 3, 5, and 7 days after rhG-CSF injection, 20 μL of blood were taken from the orbital venous plexus. White blood cells (WBC) in peripheral blood were tested by an automatic biochemical analyzer (Hitachi Ltd., Tokyo, Japan).

Histological analysis

Animals were sacrificed after 3 or 6 weeks of treatment, the tibia and femur were dissected, fixed in 4% paraformaldehyde for 24 h, decalcified in 10% ethylene diamine tetraacetic acid-2Na for decalcification, and embedded in paraffin. Tissue sections (4 μm) were mounted on common slides for staining with hematoxylin and eosin (HE), toluidine blue, and Masson's trichrome, as described.[11,12] Cartilage histopathological features were analyzed using the scoring system by Mankin *et al* [13] (score ranging from 0-12 for normal to complete disorganization and hypocellularity). Synovium histopathology was evaluated according to Yoshi-

mi's histological grading (score ranging 0-18 for normal to most severe reaction).[14] Image-Pro Plus 6.0 System (Media Cybernetics Inc., Rockville, MD, USA) image analysis system was used for quantitative analysis. The positive index was calculated as integrated optical density (IOD). All sections were randomized and evaluated by a trained observer who was blinded to the treatment groups.

Terminal deoxynucleotide transferase-mediated dUTP nick-end labeling (TUNEL) staining

Apoptotic cells in specimens were recognized using in situ cell apoptosis detection kits (Boster) according to the manufacturer's instructions.[15] Briefly, deparaffinized sections were permeabilized in 0.1% Triton X-100, incubated in fluorescein-labeled dUTP and terminal deoxynucleotidyl transferase (TdT) mixture, then probed using anti-fluorescein antibody conjugated with alkaline phosphatase. Sections were developed using a substrate solution containing fast red. Sections without primary TdT were used as negative controls for the TUNEL staining. TUNEL-positive cells in three different areas were counted under a microscope. Image-Pro Plus 6.0 System (Media Cybernetics Inc., Rockville, MD, USA) image analysis system was used for quantitative analysis.

Immunoblotting analysis

Paraffin sections (4 μm) of joint tissues were mounted on poly-L-lysine-coated slides. Paraffin sections were routinely dewaxed and incubated for 10 min with 3% H_2O_2. Each section was incubated with blocking serum (Vectastain ABC Kit, Vector Laboratories, Burlingame, CA, USA) at room temperature for 30 min and then with primary rabbit monoclonal antibody against type Ⅱ collagen (dilution 1∶30), rabbit polyclonal antibody against MMP-13 (dilution 1∶50), rabbit monoclonal antibody against TIMP-1 (dilution 1∶80), mouse monoclonal antibody against Brdu (dilution 1∶100), rabbit polyclonal antibody against CD34 (dilution 1∶100), and rabbit polyclonal antibody against SDF-1 (dilution 1∶50) overnight at 4℃. Sections incubated in phosphate-buffer saline (PBS) without antibody served as negative controls. Then, slides were incubated with biotinylated secondary antibody and then incubated with avidin-biotin complex reagent containing horseradish peroxidase for 30 min. Sections were then stained with 3,3'-diaminobenzidine (DAB) (Sigma, St. Louis, MO, USA). Image-Pro Plus 6.0 System (Media Cybernetics Inc., Rockville, MD, USA) image analysis system was used for quantitative analysis.

Serum and analysis

Animal blood was collected from the abdominal aorta and levels of serum interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α), bone morphogenetic protein-2 (BMP-2), transforming growth factor beta 1 (TGF-β1) and inducible nitric oxide synthase (iNOS) were analyzed by enzyme-linked immunosorbent assay or radioimmunoassay assay. Nitric oxide (NO) was detected by assay kit (Beijing 4A Biotech Co., Ltd., Beijing, China).

Statistics analysis

All data are expressed as mean±standard deviation and were calculated using a two tailed student's *t*-test, two-sample assuming unequal variance, within the Microsoft Excel 2007 software package from Microsoft (Redmond, WA, USA) Values were considered significantly different if $P<0.05$.

RESULTS

Mobilization of BMSCs

After rhG-GSF injection, WBC count in peripheral blood increased, and WBC count increased until reaching its peak on the 7th day. However, there were no significant differences among the four groups (all $P>0.05$) (Figure 1). This suggested that the application of

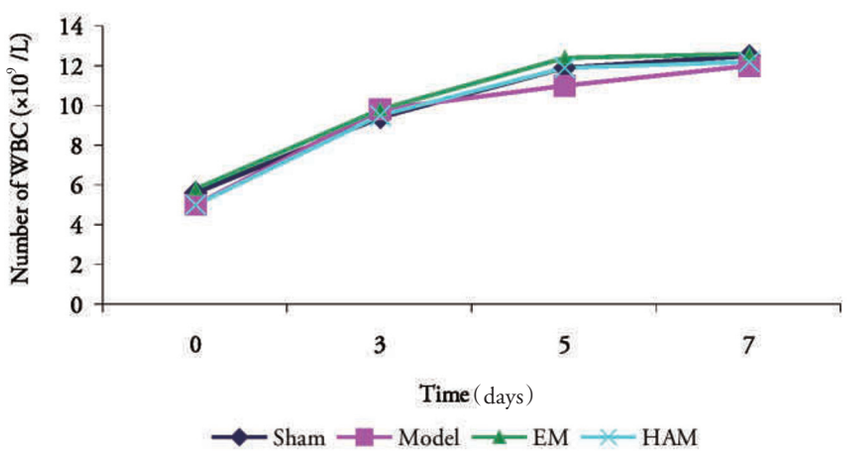


Figure 1 Comparison of WBC count among groups before and after rhG-CSF Injection
Model group: ACLT+MMx without treatment (*n*=12); sham group: sham operated (*n*=12); EM group: ACLT+MMx rats treated with EM intragastrically at a daily dose of 1.6 g/kg (*n*=12); EM plus HAM group: ACLT+MMx rats treated with EM (1.6 g/kg) plus HAM (0.3 g/kg) at a daily dose of 1.9 g/kg for 3 or 6 weeks (*n*=12). WBC: white blood cells; rhG-CSF: recombinant human granulocyte colony-stimulating factor; EM: Ermiao Fang; HAM: Xixin (*Herba Asari Mandshurici*); ACLT+MMx: anterior cruciate ligament transection and medial meniscus resection.

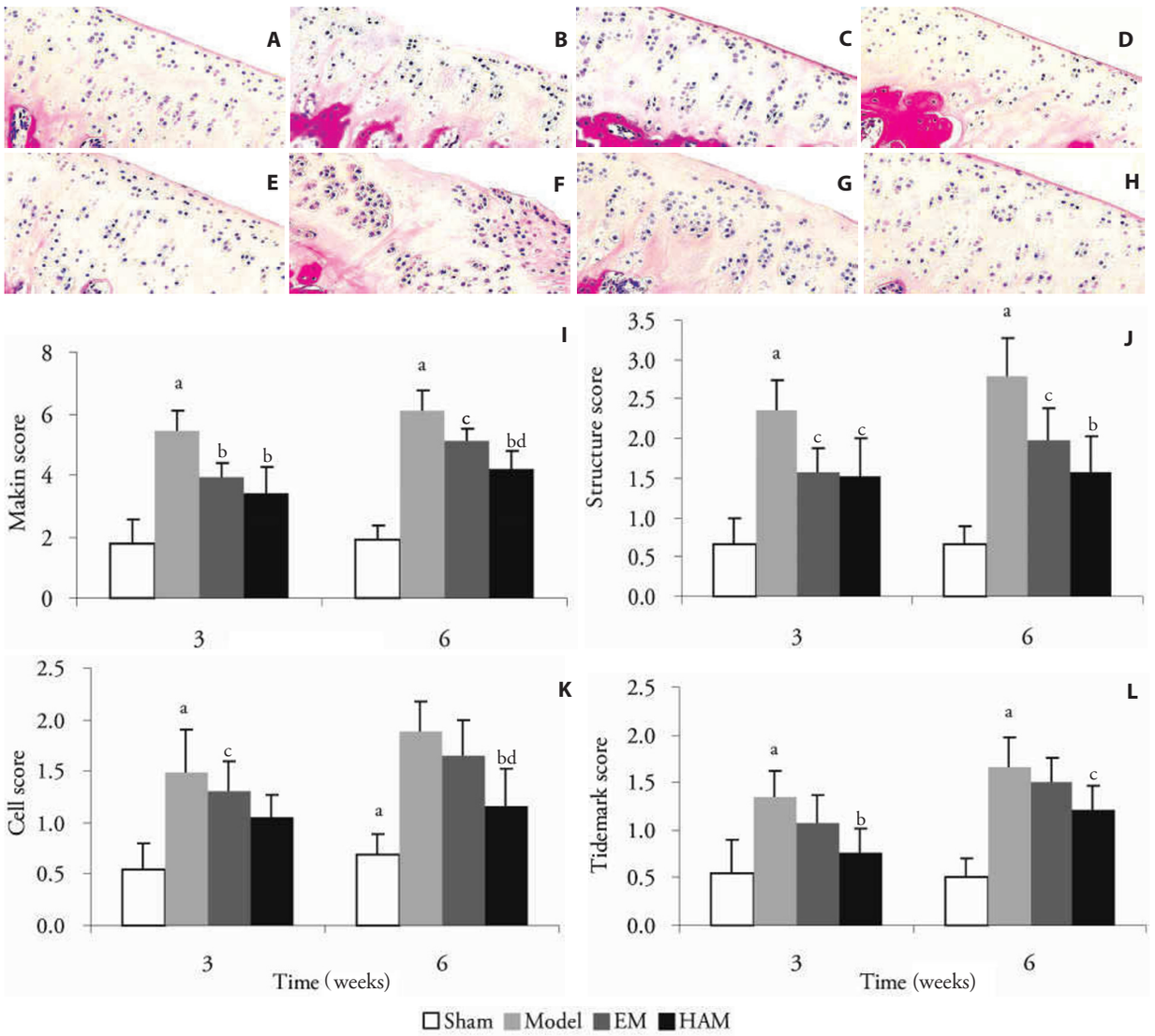


Figure 2 Comparison of effects on cartilage histology and Mankin's score

A-H: histopathologic changes of cartilage in weeks 3 and 6 (HE staining ×200). A-D: histopathologic changes of Sham, Model, EM and EM plus HAM in week 3; E-H: histopathologic changes of Sham, Model, EM and EM plus HAM in week 6. I-L: the Makin's score of cartilage, the scores of structure, cell and tidemark in weeks 3 and 6. Model group: ACLT+MMx without treatment (*n*=12); sham group: sham operated (*n*=12); EM group: ACLT+MMx rats treated with EM intragastrically at a daily dose of 1.6 g/kg (*n*=12); EM plus HAM group: ACLT+MMx rats treated with EM (1.6 g/kg) plus HAM (0.3 g/kg) at a daily dose of 1.9 g/kg for 3 or 6 weeks (*n*=12). HE: hematoxylin and eosin; EM: Ermiao Fang; HAM: Xixin (*Herba Asari Mandshurici*); ACLT+MMx: anterior cruciate ligament transection and medial meniscus resection. [a]*P*<0.01, compared with the sham group. [b]*P*<0.01 and [c]*P*<0.05, compared with the model group. [d]*P*<0.05, compared with the EM group.

rhG-CSF was effective in mobilizing BMSCs to peripheral blood.

Pathologic changes of articular cartilage and synovium

Figure 2 shows in the sham group, HE staining showed a smooth surface of the articular cartilage with normal cellularity. In contrast, ACLT+MMx rats had significant histopathological changes with a significantly increased overall Mankin score compared with sham rats (*P*<0.01), as indicated by surface irregularity, disorganization of articular cartilage with apparent cloning of chondrocytes in the transitional and radial zones, and disrupted tidemark. These histomorphological changes in the cartilage were reduced in the EM and EM plus HAM- Medtreated rats. Meanwhile, the overall Mankin scores of the two Traditional Chinese Medicine groups were significantly lowered as compared with those of the model group at 3 and 6 weeks treatment (*P*<0.01 or *P*<0.05). Notably, EM plus HAM significantly lowered the overall Mankin scores compared with EM at 6 weeks (*P*<0.05).

Untreated ACLT+MMx rats had more hyperplasia of synovial lining cells, hypertrophy of synovial lining layer, and growth of the infiltration of inflammatory cells in synovial tissue compared with sham rats. EM and EM plus HAM rats had less hypertrophy of the synovial lining layer, inflammatory cellular infiltration, and had significantly lower histological severity scores compared with untreated ACLT+MMx rats after 6 weeks of treatment (*P*<0.05 or 0.01). The largest inflammation suppression was found in the EM plus HAM

group. However, there were no significant differences between the EM and EM plus HAM groups. (Figure 3)

Chondrocyte apoptosis

Figure 4 shows that there were many apoptotic chondrocytes in the cartilage of untreated ACLT+MMx rats in weeks 3 or 6. EM and EM plus HAM rats had significantly lower percentages of apoptotic chondrocytes in weeks 3 or 6. The EM plus HAM group had 35% fewer apoptotic chondrocytes compared with EM treatment in week 6 (P<0.01).

Cartilage matrix repair

Untreated ACLT+MMx rats had significantly less proteoglycan, collagen, and type Ⅱ collagen in weeks 3 or 6. The EM and EM plus HAM groups had significantly higher levels of proteoglycan and collagen and more expression of type Ⅱ collagen in week 6 compared with the model group. EM plus HAM group had a 1.2-, 1.3- and 1.3-fold increase in respective proteoglycan, collagen and type Ⅱ collagen expressions, compared with the EM treatment group in week 6 (all P< 0.05) (Figure 5).

Expressions of MMP-13 and TIMP-1 in articular cartilage

Figure 6 shows that ACLT + MMx induced a significant increase in MMP-13 expressions and a clear decrease of TIMP-1 expressions in the joints in weeks 3 or 6. EM and EM plus HAM treatment showed significant differences after 6 weeks of treatment. EM plus HAM had 15.9% less positive MMP-13 expression and 26.9% more TIMP-1 expression compared with the EM group in week 6 (all P<0.05). MMP-13 and TIMP-1 were expressed in the chondrocytes of the knee joint in all groups. The positive staining was predominantly cytoplasmic or cytosolic in the chondrocytes.

Expressions of BrdU, CD34, and SDF-1 on chondrocyte in articular cartilage

Compared with the sham-operated group, the number of BrdU-positive cells in the model group was higher both in weeks 3 and 6, but there were no statistical differences (P>0.05). Compared with the untreated

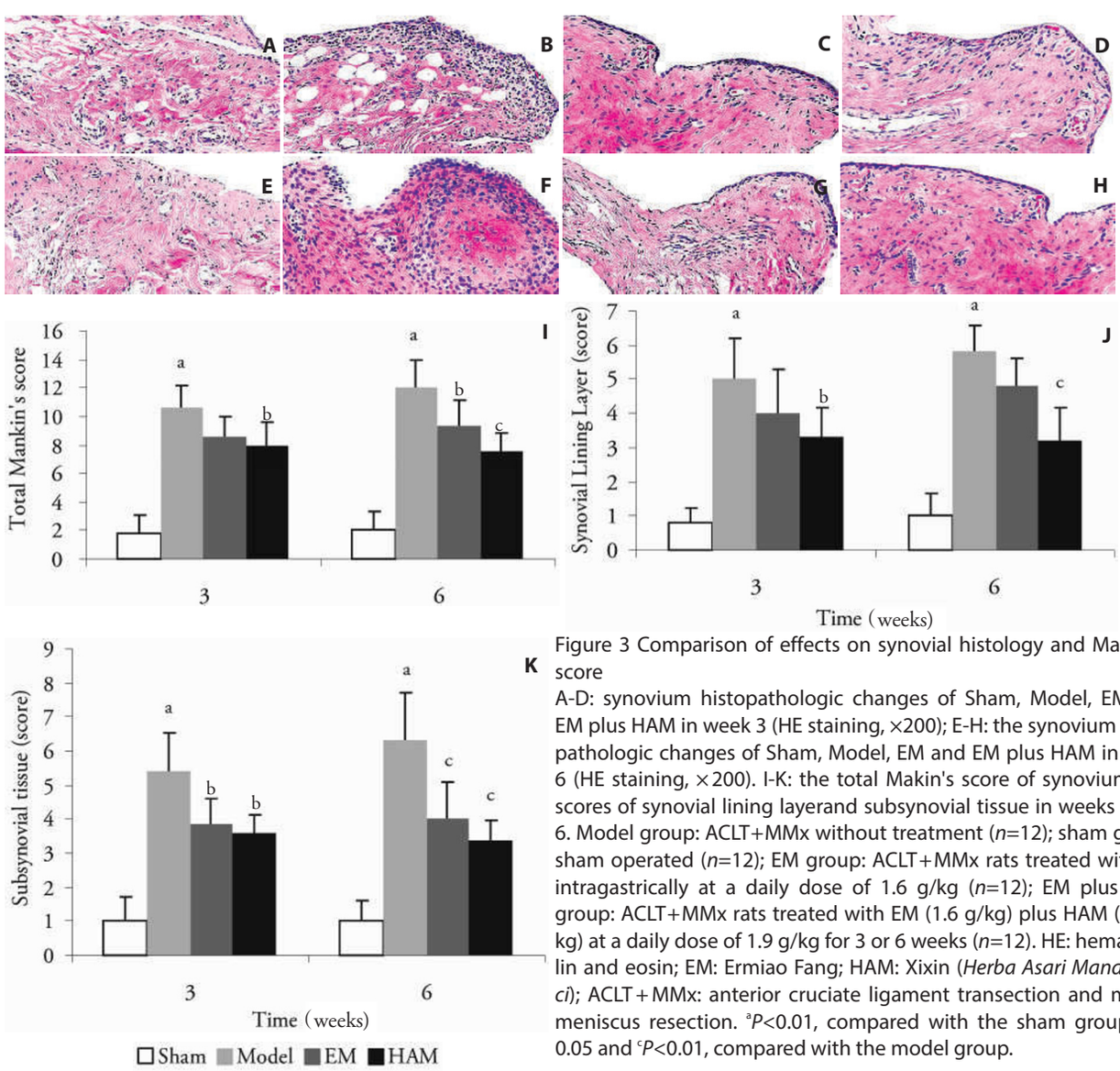
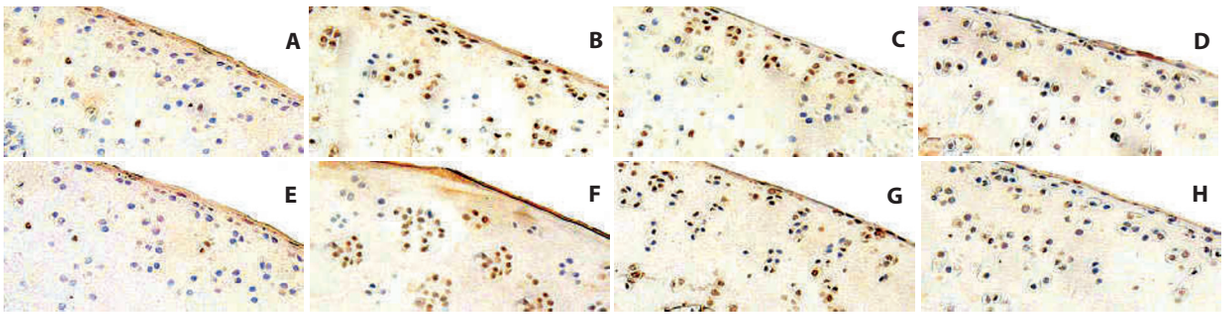


Figure 3 Comparison of effects on synovial histology and Mankin's score

A-D: synovium histopathologic changes of Sham, Model, EM and EM plus HAM in week 3 (HE staining, ×200); E-H: the synovium histopathologic changes of Sham, Model, EM and EM plus HAM in week 6 (HE staining, ×200). I-K: the total Makin's score of synovium, the scores of synovial lining layerand subsynovial tissue in weeks 3 and 6. Model group: ACLT+MMx without treatment (n=12); sham group: sham operated (n=12); EM group: ACLT+MMx rats treated with EM intragastrically at a daily dose of 1.6 g/kg (n=12); EM plus HAM group: ACLT+MMx rats treated with EM (1.6 g/kg) plus HAM (0.3 g/kg) at a daily dose of 1.9 g/kg for 3 or 6 weeks (n=12). HE: hematoxylin and eosin; EM: Ermiao Fang; HAM: Xixin (*Herba Asari Mandshurici*); ACLT + MMx: anterior cruciate ligament transection and medial meniscus resection. ^{a}P<0.01, compared with the sham group. ^{b}P< 0.05 and ^{c}P<0.01, compared with the model group.



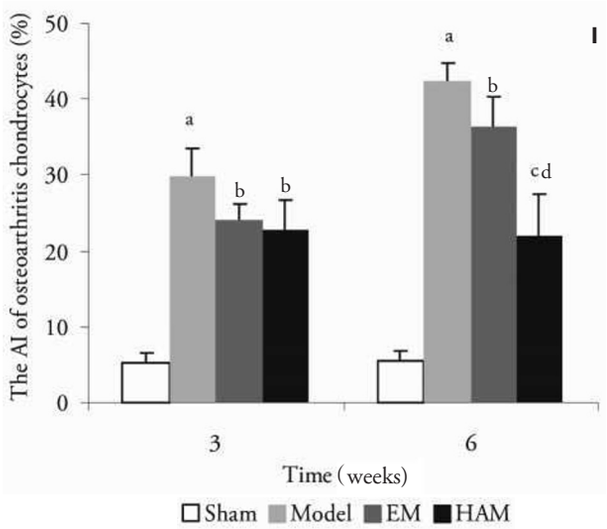


Figure 4 Comparison of effects on chondrocytes apoptosis in weeks 3 and 6

A-H: terminal deoxynucleotide transferase-mediated dUTP nick-end labeling staining (×200). A-D: the chondrocytes apoptosis of Sham, Model, EM and EM plus HAM in week 3; E-H: The chondrocytes apoptosis of Sham, Model, EM and EM plus HAM at the 6 week. I: Apoptosis index of osteoarthritis chondrocytes. Model group: ACLT+MMx without treatment (n=12); sham group: sham operated (n=12); EM group: ACLT+MMx rats treated with EM intragastrically at a daily dose of 1.6 g/kg (n=12); EM plus HAM group: ACLT+MMx rats treated with EM (1.6 g/kg) plus HAM (0.3 g/kg) at a daily dose of 1.9 g/kg for 3 or 6 weeks (n=12). EM: Ermiao Fang; HAM: Xixin (*Herba Asari Mandshurici*); ACLT+MMx: anterior cruciate ligament transection and medial meniscus resection. [a]P<0.01, compared with the sham group. [b]P<0.05 and [c]P<0.01, compared with the model group. [d]P<0.01, compared with the EM group.

group, in week 3, there were significantly more positive cells in the EM plus HAM group (P<0.05). However, in the 6th week, there were significantly more positive cells in both the formulae groups, while the most cells were found in the EM plus HAM group. There was a significant difference between the EM and EM plus HAM groups (P<0.05) (Figure 7).

The number of CD34 and SDF-1-positive cells in the untreated group was significantly higher in week 3 and lower in week 6 as compared with that in the sham-operated group. EM plus HAM group had significantly greater expressions of CD34 and SDF-1 both in weeks 3 and 6 as compared with the untreated group. Only in week 6 was the CD34 and SDF-1 expression in the EM plus HAM group significantly higher than that in the EM group (P<0.05) (Figure 7).

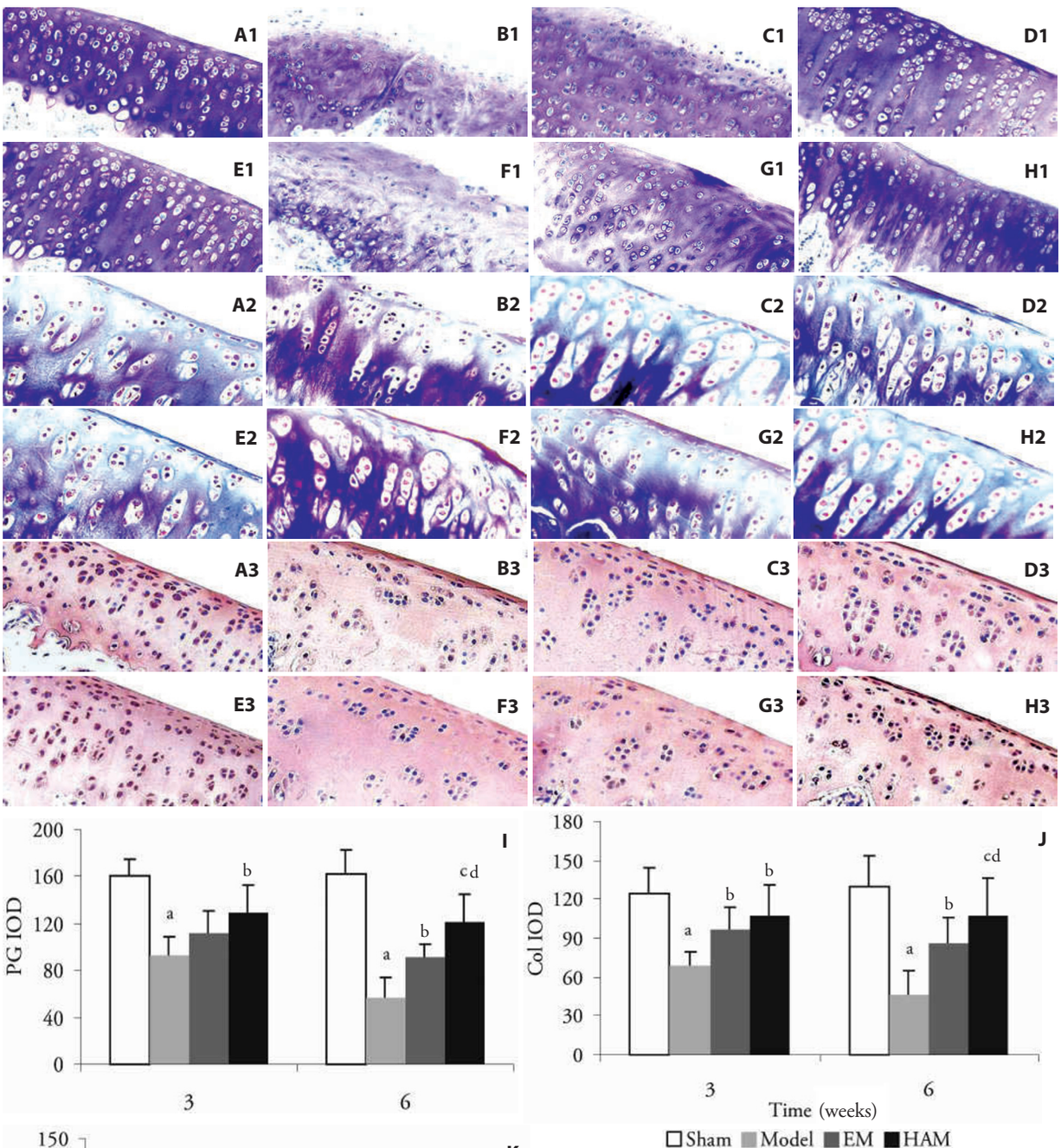
Serum IL-1β, TNF-α, NO, iNOS, BMP, and TGF-β1

Serum levels of IL-1β, TNF-α, NO, and iNOS were significantly higher, and the levels of BMP-2 and TGF-β1 were significantly lower in the untreated ACLT+MMx group compared with the sham group. EM and EM plus HAM groups had significantly lower levels of IL-1β, TNF-α, NO, iNOS in week 6. Compared with the untreated group, the levels of BMP-2 and TGF-β1 in the EM plus HAM group were significantly higher (P<0.05) in week 6. However, there were no significant differences in the above indexes between the EM and EM plus HAM groups (Figure 8).

DISCUSSION

BMSC therapy has been used in bone and cartilage repair for osteoarthritis treatment.[16] However, the limited ability of BMSCs to home and repair is the bottleneck for BMSC therapy.[17,18] Therefore, finding agents or methods that could promote BMSC homing to lesions is popular in OA treatment research. In the present study, we evaluated the anti-osteoarthritis effects of EM and EM plus HAM on the mobilization of BMSC homing to a focal zone using an ACLT+MMx rat model. We found significantly less articular cartilage damage and synovium inflammation in the EM and EM plus HAM groups than in the model group, and HAM was able to enhance the curative effects of EM on OA. Moreover, EM plus the medical guide HAM can promote directional homing of BMSCs to pathologically damaged areas.

Chondrocyte apoptosis alters the cartilage matrix (ECM) synthesis, leading to matrix degeneration and finally to OA.[19] Cartilage ECM molecules such as type Ⅱ collagen and sulfated proteoglycan play a crucial role in regulating chondrocyte functions by facilitating cell-matrix interactions.[20] The development and progression of OA is believed to involve inflammation, and IL-1β, TNF-α, and NO have been reported to induce apoptosis in chondrocytes and cause extracellular matrix degration.[21] MMP-13 can degrade type Ⅱ collagen and its activities are inhibited by TIMP-1. An imbalance in the ratio of TIMPs to MMPs causes continued matrix destruction in OA.[22] Our results demon-



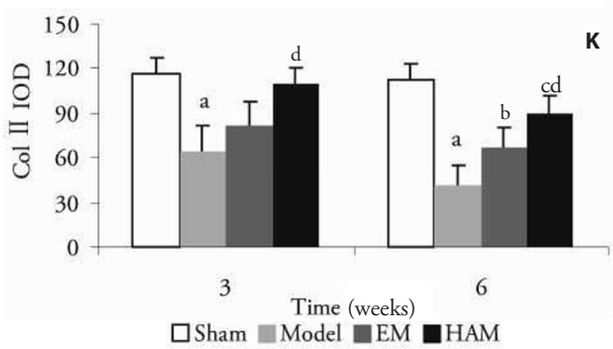


Figure 5 Comparison of effects on PG, COL, and COL Ⅱ

A1-D1: the PG of Sham, Model, EM and EM plus HAM in week 3 ; E1-H1: the PG of Sham, Model, EM and EM plus HAM in week 6 . A2-D2: the COL of Sham, Model, EM and EM plus HAM in week 3 ; E2-H2: the COL of Sham, Model, EM and EM plus HAM in week 6 . A3-D3: the COL Ⅱ of Sham, Model, EM and EM plus HAM in week 3; E3-H3: the COL Ⅱ of Sham, Model, EM and EM plus HAM in week 6. Dyeing methods of PG, COL, in week 6. A3-D3: the COL Ⅱ of Sham, Model, EM and EM plus HAM in week 3; E3-H3: the COL Ⅱ of Sham, Model, EM and EM plusHAM in week 6. Dyeing methods of PG, COL, and COL Ⅱ were toluidine blue staining, Masson's trichrome staining, and immunohistochemistry, respectively (×200). I-K: Levels of PG, COL, and COL Ⅱ. Model group: ACLT+MMx without treatment (n=12); sham group: sham operated (n=12); EM group: ACLT+MMx rats treated with EM intragastrically at a daily dose of 1.6 g/kg (n=12); EM plus HAM group: ACLT+MMx rats treated with EM (1.6 g/kg) plus HAM (0.3 g/kg) at a daily dose of 1.9 g/kg for 3 or 6 weeks (n=12). PG: proteoglycan; COL: collagen; COL Ⅱ: type Ⅱ collagen. EM: Ermiao Fang; HAM: Xixin (*Herba Asari Mandshurici*); ACLT+MMx: anterior cruciate ligament transection and medial meniscus resection. [a]P<0.01, compared with the sham group. [b]P<0.05 and [c]P<0.01, compared with the model group. [d]P<0.05, compared with the EM group.

strated that EM and EM plus HAM treatment had significantly less articular cartilage damage and synovium inflammation in OA rats in week 6 than that in the model group. Meanwhile, the two formulae groups

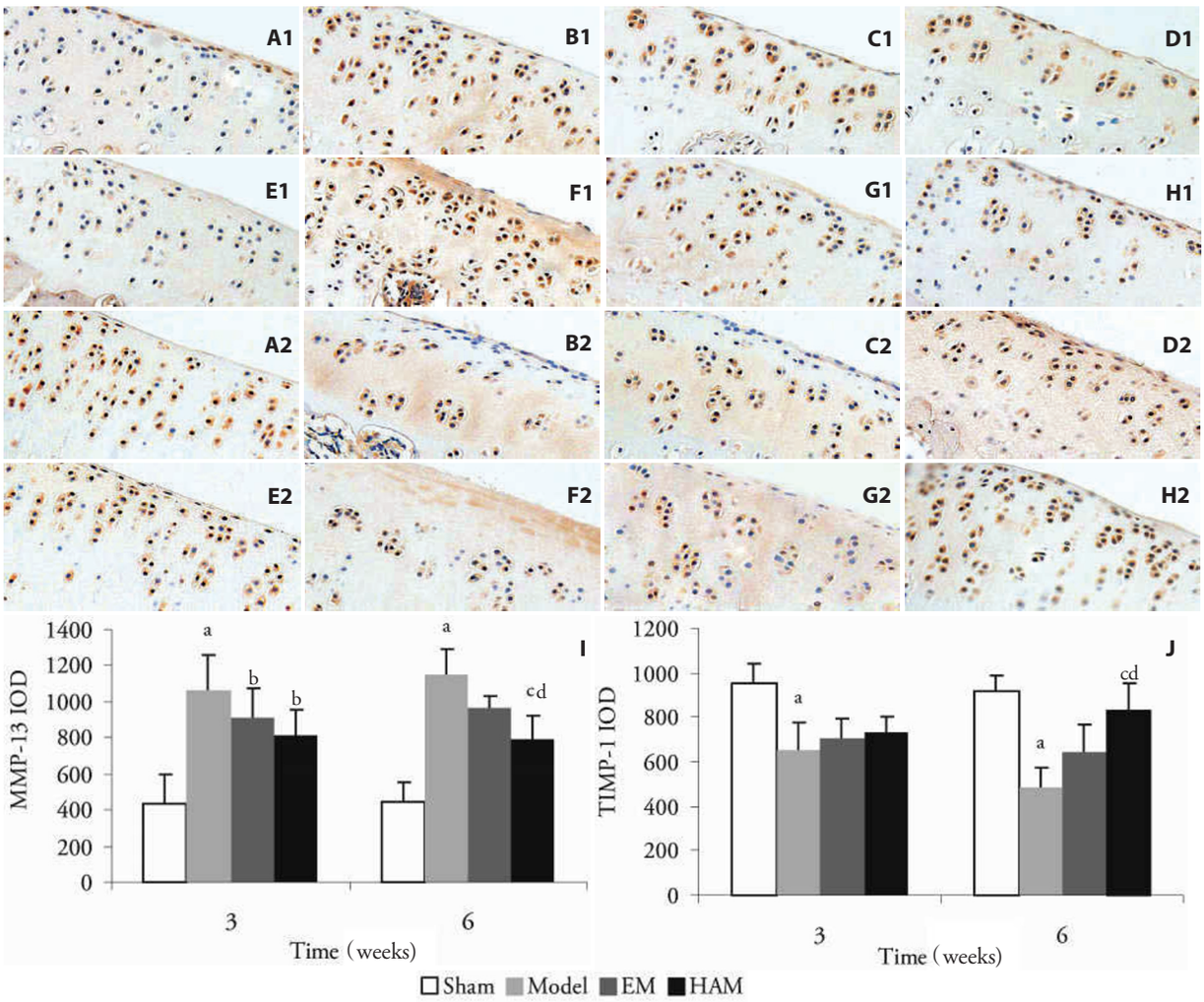
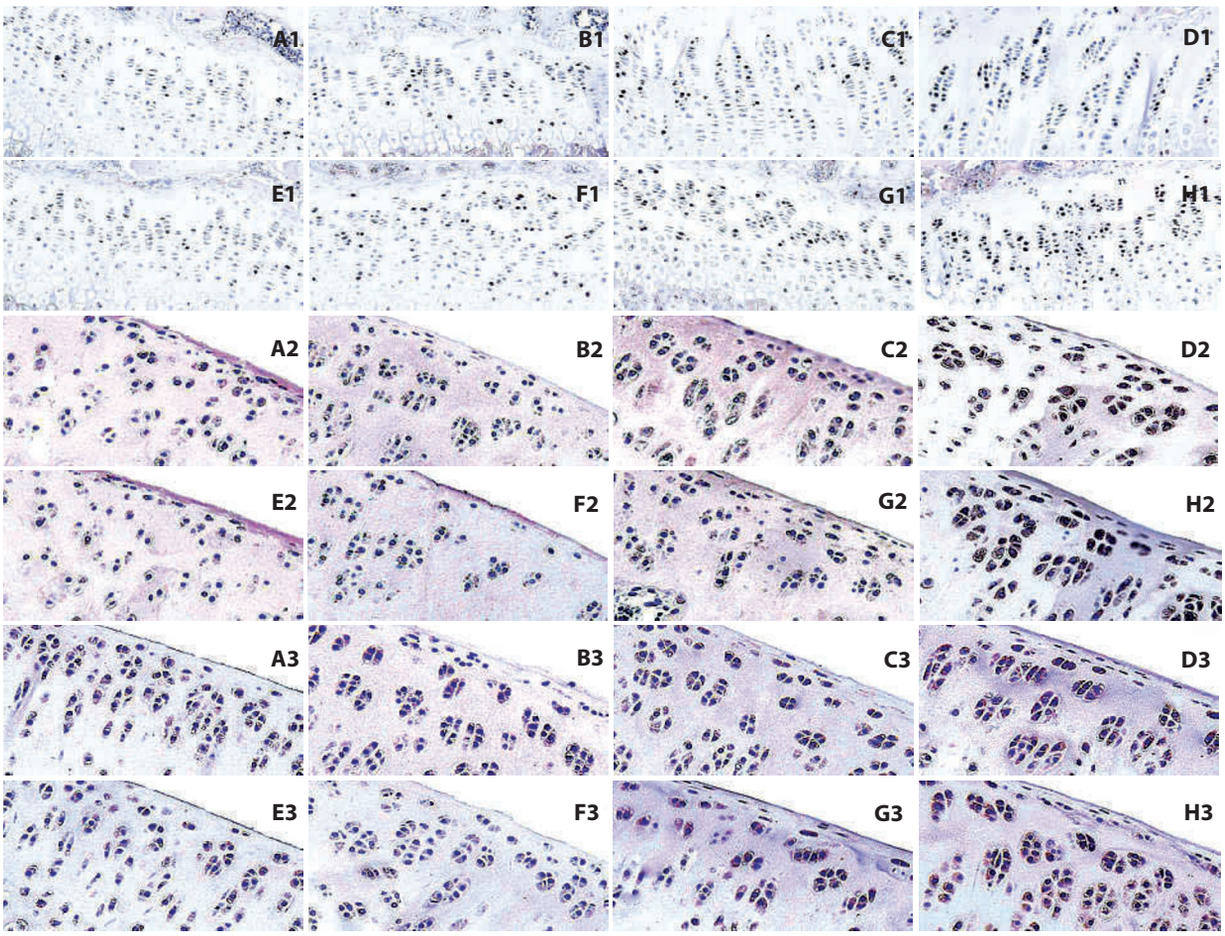


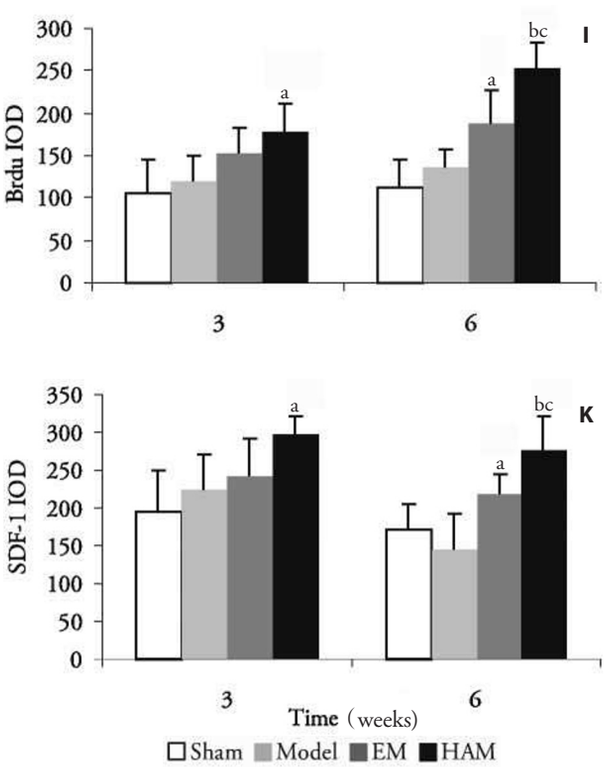
Figure 6 Comparison of expressions of MMP-13 and TIMP-1 in the knee joints (×200)

A1-D1: the MMP-13 of Sham, Model, EM and EM plus HAM in week 3; E1-H1: the MMP-13 of Sham, Model, EM and EM plus HAM in week 6. A2-D2: the TIMP-1 of Sham, Model, EM and EM plus HAM in week 3; E2-H2: the TIMP-1 of Sham, Model, EM and EM plus HAM in week 6. Immunohistochemistry was used in all frames. I and J: IOD of MMP-13 and TIMP-1. Model group: ACLT+MMx without treatment (n=12); sham group: sham operated (n=12); EM group: ACLT+MMx rats treated with EM intragastrically at a daily dose of 1.6 g/kg (n=12); EM plus HAM group: ACLT+MMx rats treated with EM (1.6 g/kg) plus HAM (0.3 g/kg) at a daily dose of 1.9 g/kg for 3 or 6 weeks (n=12). MMP-13: matrix metalloproteinases-13; TIMP-1: tissue inhibitors of metalloproteinases-1; IOD: integrated optical density; EM: Ermiao Fang; HAM: Xixin (*Herba Asari Mandshurici*); ACLT+MMx: anterior cruciate ligament transection and medial meniscus resection. ^{a}P<0.01, compared with the sham group. ^{b}P<0.05 and ^{c}P<0.01, compared with the model group. ^{d}P<0.05, compared with the EM group.

had less chondrocyte apoptosis and more proteoglycan and collagen content, particularly type Ⅱ collagen expression. Both formulae obviously interfered with OA-augmented expressions of MMPs-13 and EM plus HAM clearly augmented OA-reduced TIMPs-1 expression in the knee joints. Moreover, the two formulae groups had significantly less release of IL-1β, TNF-α, NO, and iNOS in serum and the EM plus HAM group had significantly higher BMP-2 and TGF-β1 levels. Importantly, the expressions of BrdU, CD34, and SDF-1 in articular cartilage were significantly higher than those in the model group. The largest changes in these parameters were found in the EM plus HAM group. Articular cartilage repair of EM plus HAM is better than EM in ACLT+MMx rats. Because BMSCs have chondrocyte and ECM differentiation potential, our results suggest that the activity of EM plus HAM may be related to BMSC repair therapy.

BrdU is an analogue of thymidine, which is able to incorporate into the nucleus of cells during proliferation or division.[23] Therefore, it is widely used to label stem cells. After an intraperitoneal injection with BrdU, an increasing trend of BrdU-positive expression was detected in weeks 3 and 6 in the ACLT+MMx model. This suggested that, after damage of competent cells (such as osteocytes, chondrocytes, and bone marrow cells), a self-repair mechanism was initiated, and then the activated stem cells joined in the repairing activity of the articular cartilage. Although the BrdU-positive expression of the model group was higher than that of the sham-operated group, the homing stem cells were inadequate for sufficient repair, and the histopathology featured articular cartilage damage. After treatment with the two formulae, there were more BrdU positive cells. In week 3, the positive cell count of the EM plus





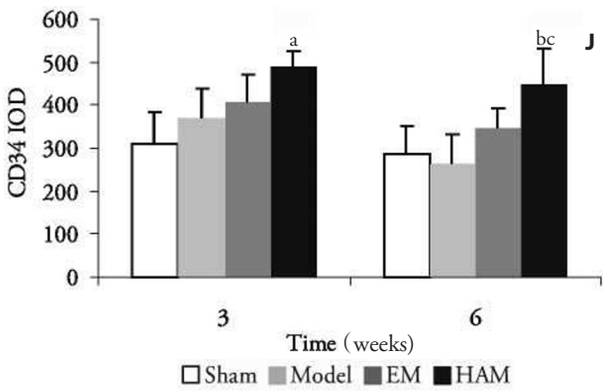


Figure 7 Comparison of expressions of Brdu, CD34, and SDF-1 in the knee joints (×200)

A1-D1: the Brdu of Sham, Model, EM and EM plus HAM in week 3; E1-H1: the Brdu of Sham, Model, EM and EM plus HAM in week 6. A2-D2: the CD34 of Sham, Model, EM and EM plus HAM in week 3; E2-H2: the CD34 of Sham, Model, EM and EM plus HAM in week 6. A3-D3: the SDF-1 of Sham, Model, EM and EM plus HAM in week 3 ; E3-H3: the SDF-1 of Sham, Model , EM and EM plus HAM in week 6. Immunohistochemistry was used in all frames (×200). I-K: IOD of Brdu, CD34, and SDF-1. Model group: ACLT+MMx without treatment (n=12); sham group: sham operated (n=12); EM group: ACLT+MMx rats treated with EM intragastrically at a daily dose of 1.6 g/kg (n=12); EM plus HAM group: ACLT+MMx rats treated with EM (1.6 g/kg) plus HAM (0.3 g/kg) at a daily dose of 1.9 g/kg for 3 or 6 weeks (n=12). BrdU: bromodeoxyuridine; CD34: cluster of differentiation 34; SDF-1: stromal cell derived factor 1; IOD: integrated optical density; EM: Ermiao Fang; HAM: Xixin (*Herba Asari Mandshurici*); ACLT+MMx: anterior cruciate ligament transection and medial meniscus resection. ^{a}P<0.05 and ^{b}P<0.01, compared with the model group. ^{c}P<0.05, compared with the EM group.

HAM group exceeded that of the model group, while the EM and model groups were not significantly different. The stem cells could possibly differentiate into chondrocytes or extracellular matrix, and there was sig-

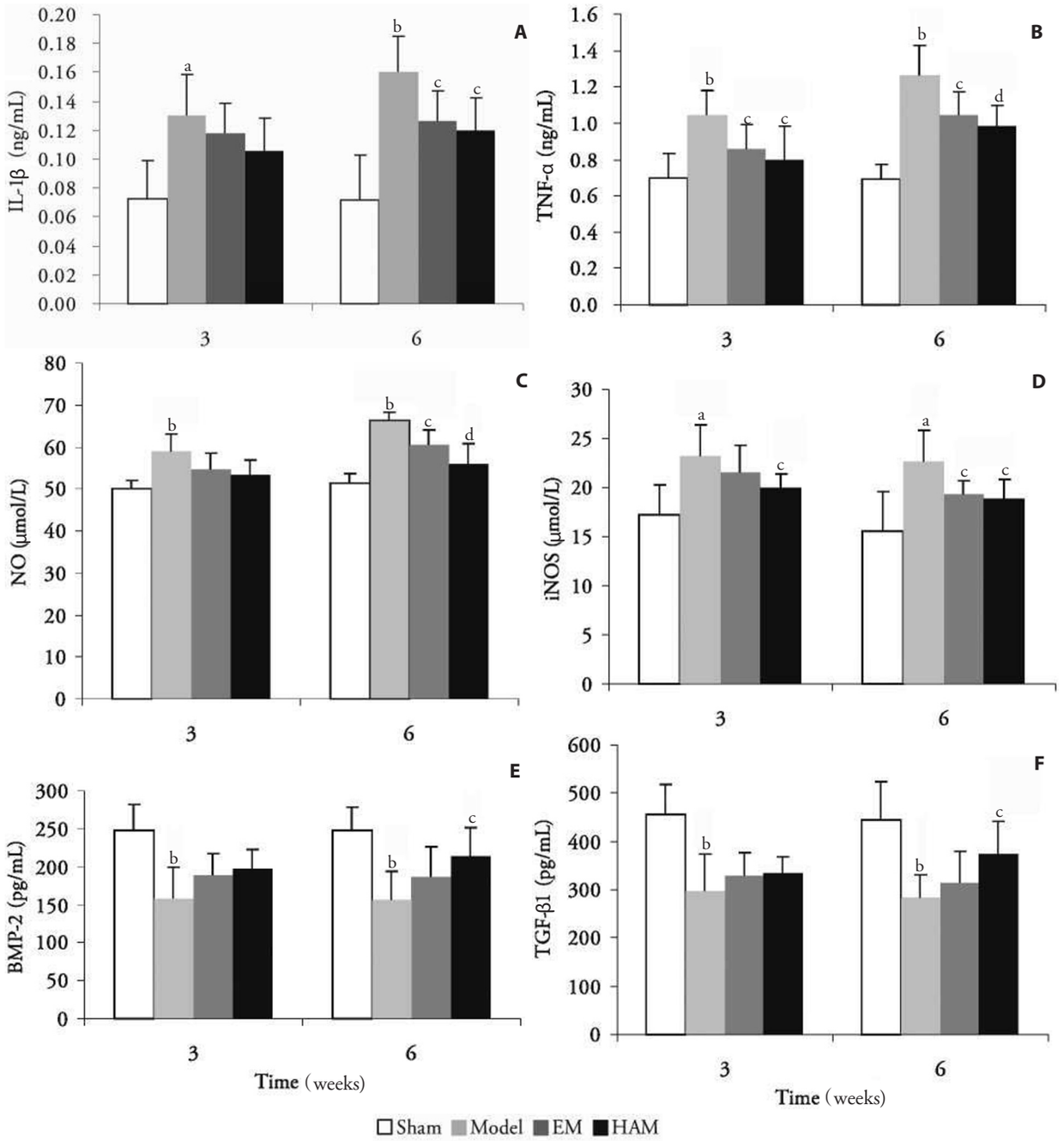


Figure 8 Comparison of effects on IL-1β, TNF-α, NO, iNOS, BMP-2, and TGF-β1 in serum

A-F: the levels of IL-1β, TNF-α, NO, iNOS, BMP-2, and TGF-β_1 in serum in weeks 3 and 6. Model group: ACLT+MMx without treatment (*n*=12); sham group: sham operated (*n*=12); EM group: ACLT+MMx rats treated with EM intragastrically at a daily dose of 1.6 g/kg (*n*=12); EM plus HAM group: ACLT+MMx rats treated with EM (1.6 g/kg) plus HAM (0.3 g/kg) at a daily dose of 1.9 g/kg for 3 or 6 weeks (*n*=12). EM: Ermiao Fang; HAM: Xixin (*Herba Asari Mandshurici*); ACLT+MMx: anterior cruciate ligament transection and medial meniscus resection. Data represent the mean±*SD* of samples with 6 rats in each. [a]*P*<0.05, [b]*P*<0.01 compared with the sham group. [c]*P*<0.05 and [d]*P*<0.01, compared with the model group.

nificantly more repair observed in the articular cartilage of both formulae groups. In addition, the articular cartilage Mankin score of the EM plus HAM group was markedly lower than that of the EM group. This indicated that HAM could further promote stem cell homing and the initiation of homing was relatively earlier.

The CD34 molecule is a transmembrane salivary mucin, and is considered to be a positive sign of hematopoietic stem cells.[24] In this study, we found that the CD34 expression in week 6 was lower than that in week 3. The reason was possibly because the differentiation of activated stem cells occurred in week 6. EM is able to slightly up-regulate the positive expression of CD34, and this effect was enhanced after addition of HAM. This difference was clearly observed in terms of CD34 level in week 6, which is consistent with the significantly higher BrdU-positive cell count at the same time. These results suggested that HAM might promote BMSC homing to the damage area.

SDF-1 is the first reported chemoattractant protein that participates in the homing course of stem and pro-

genitor cells. Furthermore, the homing of stem cells is dependent on the SDF-1 concentration gradient.[25] In this study, EM was able to significantly up-regulate the positive expression of SDF-1 and thereby facilitate BMSC homing activity. This effect was significantly enhanced after addition of HAM in week 6, which is consistent with the significantly higher BrdU-positive cell count at the same time. Therefore, HAM might promote BMSC homing to damage by increasing SDF-1 levels in pathologic tissues and consequently boosting the repair activity.

In conclusion, EM and EM plus HAM exert significant anti-osteoarthritis effects in ACLT+MMx rats. Importantly, by studying the influence of EMR plus HAM (a medicinal guide) on BMSCs homing to damage focus and repair function, we primarily expound the mechanism of HAM in enhancing curative effects of EM for OA may be by promoting the homing of BMSCs of peripheral blood to the damage focus. The facilitation of BMSC homing to pathologic areas could be the biological manifestation of HAM guiding other drugs to a focal zone.

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