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Original Article

Effect of semisynthetic extracellular matrix-like hydrogel containing hepatocyte growth factor on repair of femoral neck defect in rabbits

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Abstract: Using tissue engineering technology research to develop organized artificial bone, then repair bone defect. This work aims to investigate the role of semisynthetic extracellular matrix-like hydrogel (sECMH) containing hepatocyte growth factor (HGF) on repair of femoral neck defect in rabbits. 18 New Zealand rabbits were used in this study. According to autologous paired comparison method, the left and right sides of rabbit were used as control and experimental side, respectively. The models of bilateral femoral neck bone defect were established. In experimental side, sECMH containing HGF was implanted in the defect area. In control side, no material was implanted in the defect area. At the 2nd, 4th and 8th week after surgery, the gross observation, histological examination and molybdenum target (Mo-target) X-ray examination were performed on the specimens to study the repair of femoral neck defect. In gross observation, there was no macroscopic difference of femoral neck specimen between the 2nd and 4th postoperative week. At the 8th week, the defect orifice was closed with immature cortical bone, with unblocked marrow cavity. HE staining results showed that, at the 4th week, there were more new vessels in defect area of experimental side, compared with control side. At the 8th week, in experimental side there was immature cortical bone connecting the fracture end in defect area, with visible bone marrow cells. Mo-target X-ray examination found that, at the 8th week, the bone tissue repair in experimental side was better than control side. As a new drug delivery system, sECMH containing HGF has good application prospect in bone tissue repair.

Keywords: Hydrogel, hepatocyte growth factor, bone defect, repair

Introduction

Bone defect is a clinically common factor leading to disability. The infection in surgery, tumor, trauma, osteomyelitis and congenital diseases are the main causes of bone defect [1]. Bone grafting is the traditional method commonly used for treatment of bone defect, which has obtained certain therapeutic efficacy. Khosla [2] has firstly conducted the repair of mandibular defect with vascularized rib graft. Davis et al. [3] have also performed the vascularized rib graft on animals. However, the application of bone graft still has many restrictions. All autogenous bone grafting materials are obtained in surgery, which will inevitably lead to extension of surgical time, aggravation of patient pain, and increase of infection chance. At the same time, some postoperative complications often appear, including chronic donor-site pain, infection, hematoma, deformity, pressure fracture, and skin hypoesthesia [4]. In addition, the bone source of autologous bone graft is limited, and the graft bone morphology and size are not easy to meet the requirements. The tissue-engineered bone prepared using tissue engineering technology has advantages incomparable for grafting bone [5]. At present, continued improvement of a variety of ideal bone materials and development of bone tissue engineering are the research emphasis.

Hepatocyte growth factor (HGF), also called discrete factor, is derived from the mesenchymal tissue, and can act with various growth factors originating from mesenchymal tissue. It can promote the angiogenesis, and enhance the expression of bone morphogenetic protein (BMP) receptor [6], thus promoting the bone tissue regeneration. Semisynthetic extracellular

matrix-like hydrogel (sECMH) is prepared by cross-linked reaction from thiol-modified hyaluronic acid and thiol-modified gelatin [7, 8]. It can be used as a transmission system to combine a variety of cytokines, and slowly release the cytokines [7, 9, 10]. In vitro studies [11] show that, sECMH containing HGF can slowly release HGF. In this study, sECMH containing HGF was implanted in femoral neck defect in rabbits, and its effect on bone tissue repair was observed.

Materials and methods

Preparation of sECMH

Thiol-modified hyaluronic acid and thiol-modified gelatin were mixed under aseptic condition according to product specification, followed by shaking. According to method reported by Zhao et al. [12], HGF (PeproTech Asia Inc., USA) was added to the mixture (2.5 μg of HGF for 1 mL of mixture), followed by addition of polyethylene diacrylate solution. After shaking, the final mixture was added to aseptic EP tubes, 0.1 mL of mixture for each tube, followed by 30 m of standing at room temperature for cross-linked reaction. The prepared sECMH was preserved at -20°C for use.

Establishment of animal models of femoral neck bone defect

18 New Zealand rabbits (pure line, clean grade, 6-8 months old, 2.8 ± 0.7 kg, regardless of gender) were enrolled in this study. Before experiments, the rabbits were reared in single cage and fed with granule feedstuff, with free access to water. The disposal on animals met the ethical standards in animal experiments. According to autologous paired comparison method, the left and right sides of rabbit were used as control and experimental side, respectively.

Rabbits were anaesthetized by intravenous injection with 3% pentobarbital sodium. After disinfection, the incision was made on posterolateral right hip joint. The skin and subcutaneous superficial fascia were incised in turn, followed by relieving of deep fascia. The fascia between quadriceps and gluteus maximus was incised, followed by tongue-shaped incision of rear gluteus mediums and resection of rear gluteus minimus. The piriformis tendon was incised, followed by cross incision of hip joint capsule and full relieving of rear femoral neck. A 3.5 mm drill bit was used to drill from the center of rear femoral neck to contralateral femoral neck cortex, and then the 4 mm drill bit was used for reaming. The cancellous bone and bone marrow in internal and external marrow cavity were removed using a small curette. So a backward sinus-shaped femoral neck defect with 4 mm diameter was established. sECMH 0.1 ml containing 0.25 μg HGF was implanted in the defect area. Finally, the joint capsule was sutured, followed by layer-by-layer suture of muscle, fascia and skin. The model establishment method in left side was the same with right side, followed by joint capsule suture without any treatment in defect area. After surgery, the rabbits were treated by intramuscular injection with gentamicin (40000 U/rabbit) for 3 consecutive days, then were reared in single cage and fed with routine feedstuff, with voluntary activities.

General observation

During the experimental period, the survival, eating situation, resting posture, movement, posture and activities of rabbits were observed, and the reddish swelling, infection, liquid oozing and abscess in surgical site were observed.

Gross observation

At the 2nd, 4th and 8th week after surgery, 3 rabbits were executed in each group. According to original surgical approach, the skin and subcutaneous tissue was incised layer-by-layer, and the scar tissue was separated. The inflammation response in tissue around defect area was observed. The central defect site was opened, and the soft tissue around defect area and repair of defect were observed.

Histological examination

At the 2nd, 4th and 8th week in experiment, the femoral neck specimen with femoral head of was taken from executed rabbit, followed by routine fixation, decalcification, dehydration, and embedding. After chipping specimen to middle sagittal plane of bone defect, 3 consecutive slices were made. After HE staining, the slices were observed by 2 pathologists with more than 10 years of experience in Affiliated Zhongshan Hospital of Dalian University for randomized and double-blind evaluation. The

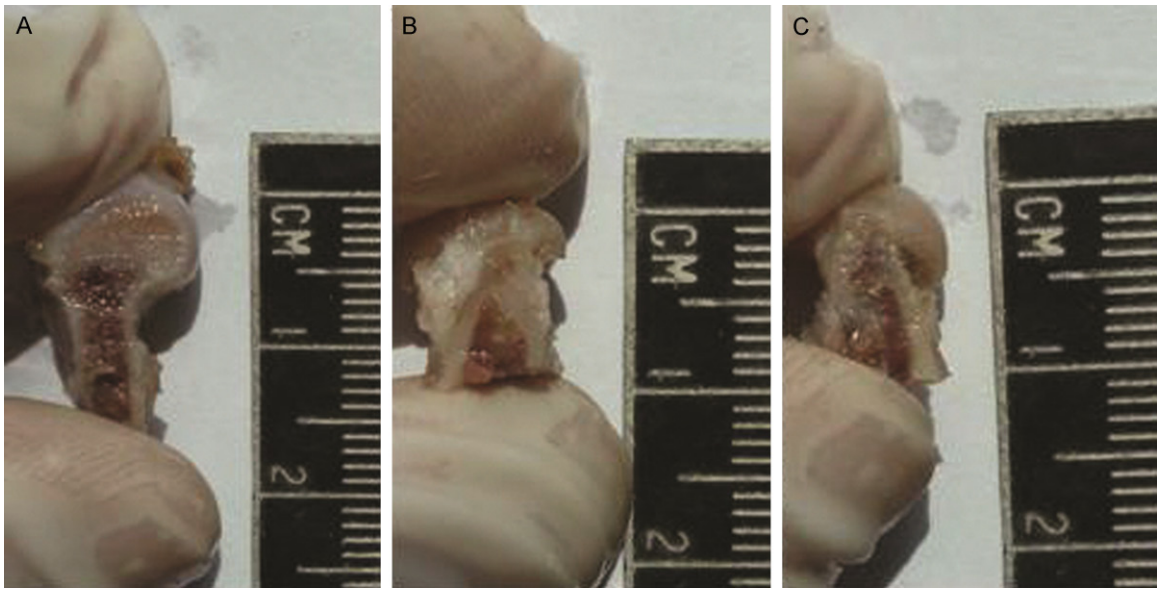

Figure 1. Gross observation results. A. Normal rabbit femoral neck; B. Femoral neck in control side at the 8th postoperative week; C. Femoral neck in experimental side at the 8th postoperative week.

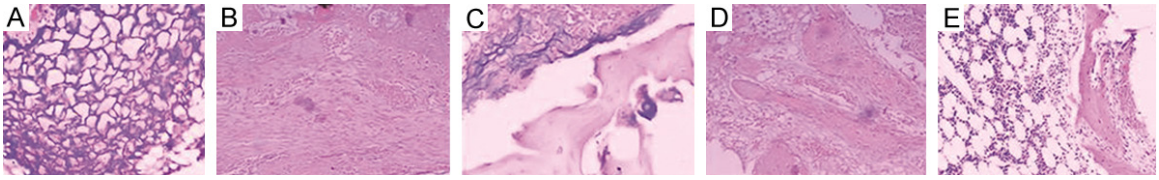

Figure 2. A. sECMH (blue) in experimental side at 2nd postoperative week (× 100). B. Experimental side at 2nd postoperative week (× 100); C. sECMH (blue) in experimental side at 4th postoperative week (× 100); D. Experimental side at 4th postoperative week (× 100); E. Experimental side at 8th postoperative week (× 100).

bone trabecula, osteocytes, osteoblasts, osteoid, and angiogenesis, and sECMH absorption in bone tissue were observed.

Radiological examination

At the day of surgery and at the 4th and 8th week after surgery, the Mo-target X-ray examination was performed on both sides of hip. All radiographic films were evaluated in randomized and double-blind conditions by 2 radiologists with more than 10 years of experience to analyze the repair of bone defect.

Results

General observation

After surgery, there was no reddish swelling, infection, liquid oozing or abscess in incision in all rabbits, with good apposition of skin flap. At the 2nd postoperative week, the incision was healed. At the 4th week, all sutures fell off naturally. The rabbit fur was shiny and supple, with erection of both ears. Rabbits had frequent nocturnal activities, with good eating situation. No rabbit died unexpectedly after surgery.

Gross observation

At different time points in experiment, there was no inflammatory lesion in tissue around defect area in experimental and control side, respectively. During the experiment, the manifestations of femoral neck both in experimental and control side were different from normal femoral neck in which normal marrow cavity and cortical bone were visible (**Figure 1A**). At the 2nd and 4th postoperative week, the defect area was filled with a large amount of white fiber and fibrous callus, respectively, with unclear boundary with surrounding normal bone. The fiber and fibrous callus touched flex-

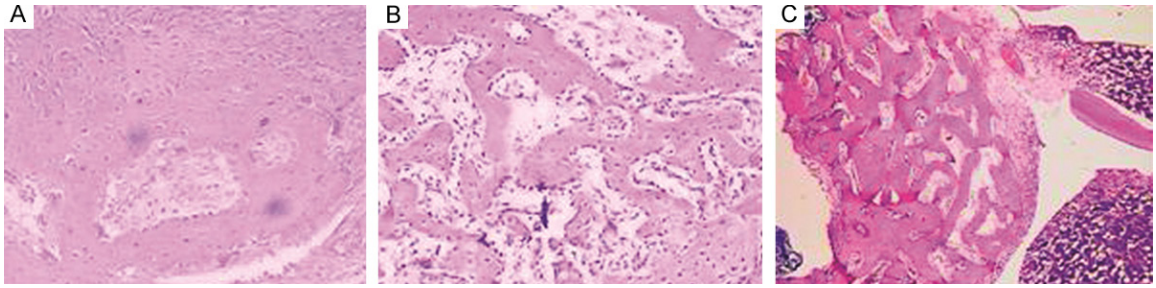

Figure 3. A. Control side at 2nd postoperative week (× 100); B. Control side at 4th postoperative week (× 100); C. Control side at 8th postoperative week (× 100).

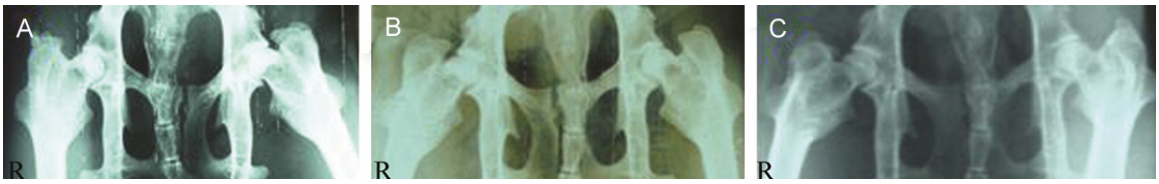

Figure 4. Mo-target X-ray examination on right hip joint of rabbit. A. The day of surgery; B. The 4th postoperative week; C. The 8th postoperative week.

ible, with no obvious resistance for pin piercing. There was no significant difference between the experimental and control side.

At the 8th week, in the bone defect area of control side, the boundary between new bone and surrounding normal bone was clear. The normal bone edge was slightly disheveled, with resistance for pin piercing. The marrow cavity was filled with a large amount of bone callus (**Figure 1B**). In experimental side, the defect orifice was closed with new bone smoothly extending from surrounding normal bone, with attachment of a small amount of fibrous tissue. The boundary between new bone and normal bone was not clear, with obvious resistance for pin piercing. The reconstructed cortical bone and marrow cavity were visible. The cortical bone in defect area was slightly thinner than surrounding normal cortical bone, with unblocked marrow cavity (**Figure 1C**).

Histological examination results

At the 2nd postoperative week, in defect area of experimental side, there was large amount of fibrous tissue surrounding the porous structure of sECMH (blue), with no cell growth at the center of sECMH (**Figure 2A**). There were rich new vessels in fibrous tissue. The mesenchymal cell hyperplasia was visible in basal part of defect area, with abundant cytoplasm and active function (**Figure 2B**). At the 4th week, there was a small amount of sECMH multiple-gap structure in 3 rabbits (**Figure 2C**). The sECMH edge was tightly combined with surrounding tissue. In remained rabbits, no sECMH structure was visible in experimental side. There was uniformly distributed osteoid in fibrous tissue around sECMH, which was connected with each other and disarranged, with monolayer-arranged active osteoblasts around. In osteoid there were many irregularly arranged osteocytes which filled the whole osseous lacuna, with a large amount of bone matrix around. The vascular structure at osteoid edge was visible, with integrate vessel wall and cell filling (**Figure 2D**). At the 8th week, there was no sECMH structure in defect area of experimental side in 12 rabbits, with unblocked marrow cavity. The marrow cavity was filled with haemocytes, adipocytes and trabecular structure along the force line. The defect orifice was closed with new cortical bone smoothly extending from surrounding normal bone. The new cortical bone was slightly thinner than surrounding normal cortical bone, with hypertrophic osteoblasts which were in functional status (**Figure 2E**).

In control side, at the 2nd postoperative week, there was a large amount of fibrous tissue in defect area, with a small number of new vessels around. There was no vascular structure in the center of defect area. The mesenchymal

cell hyperplasia in basal part of defect area was also visible, with active function (**Figure 3A**). At the 4th week, the osteoid began to appear in basal part of defect area (around fibrous tissue). With decrease of vascular structure, the amount of osteoid gradually decreased from fibrous tissue edge to the center of defect area. Compared with experimental side, the osteocyte density in osteoid in control side was higher, with thinner bone matrix around (**Figure 3B**). At the 8th week, there was wide and thick bone callus at fracture end in defect area, with active osteocytes. The trabecular connectivity was disordered, with no new cortical bone structure. The osteoblasts in bone callus had active functions, and the marrow cavity was filled with bone callus (**Figure 3C**).

Radiological examination results

At the 4th postoperative week after surgery, compared with control side, there was a lucent area in femoral neck in experimental side. There was no obvious change in defect edge in experimental side, but in control side the density of defect edge was relatively high. At the 8th week, in experimental side there was a gradual extension between defected areas and surrounding bone, and it is difficult to identify the boundary line between normal bone and new bone. In control side, the bone density at defect edge was increased, with obvious boundary with surrounding bone (**Figure 4**).

Discussion

Bone tissue regeneration is accompanied by regulation effects of multiple cytokines. HGF is initially extracted in the process of liver cell culture. The main biological functions of HGF are as follows: 1) HGF can promote the growth of liver cell and mitosis of various epithelial cells. HGF secreted by monocytes and their precursors can stimulate the growth and differentiation of bone marrow-derived hematopoietic progenitor cells. 2) HGF can directly stimulate the cell migration and invasion, up-regulate the expression of urokinase and its receptor, promote the degradation of local extracellular matrix, thus contributing to the cell invasion. 3) HGF exhibits a dual effect on endothelial cell growth and movement, and has strong pro-angiogenic effect both for normal tissue and tumor tissue [13]. 4) HGF has cytotoxic effect on tumor, with anti-apoptotic and tumor-inhibiting function. It is reported that, the HGF receptor c-met exists in the surface of bone tissue cells such as osteoclast, osteoblast, osteocyte and bone marrow mesenchymal stem cell [14-16], and has promoting or inhibiting effect on the metabolic activity of above cells. The repair of bone tissue is a comprehensive biological response process. This indicates that, HGF has great potential in bone tissue repair.

Hip joint is constituted by femoral head, femoral neck and acetabulum. The femoral neck is the arm of force. Under weight-bearing, in produces compressive stress and tensile stress in inner and outer side, respectively. In addition, it bears the corresponding shear force. The basal blood supply for femoral neck is weak. The medial and lateral arteries are the important sources of blood supply for femoral head, and their branches nourish the femoral neck. Femoral neck fracture is clinically common, and accounts for 3.58% of all fractures. It often occurs in elderly. For young, it is often manifested by violent injury, with occasional fatigue fracture. With the aging of population, there is a yearly trend of increase for femoral neck fracture in elderly due to bone quality decline [17]. Once the blood supply after fracture is destroyed or the effective blood circulation can not be timely constructed, the ischemia of fracture end will occur, with delayed healing, nonunion, and even femoral head necrosis. At the same time, the femoral neck is located in the joint cavity, without coverage of periosteum. Due to segregation of joint capsule, the femoral neck can not contact with surrounding soft tissue, thus avoiding the influence of surrounding tissue. Therefore, in this study the femoral neck is selected to construct the bone defect model for observing the effects of sECMH containing HGF on bone tissue regeneration.

Currently reported drug delivery materials include alginate, polyvinyl acetate and polylactic acid. sECMH loading HGF is a drug delivery material which can locally and sustained release HGF to exhibit its biological effect. As found in vitro experiment [12], sECMH loading HGF can slowly release HGF in vitro. The amount of released HGF within 26 d is only 35% of original loading amount. In addition, sECMH has advantages as follows: 1) Simple preparation, stable structure, and safe producing process (no use of toxic reagent). 2) High biological compatibility. 3) No immunological

rejection. 4) It can be in situ degraded in vivo, with no systemic adverse reaction. The degradation speed can be adjusted by changing the cross-linking degree of thiol-modified hyaluronic acid and thiol-modified gelatin. 5) The degraded hyaluronic acid fragments can synergistically act with the released cytokines, thus promoting the biological effect of cytokines [18].

In this study, sECMH containing HGF is implanted in femoral neck defect in rabbit. According to autologous paired comparison method, the bone tissue regeneration in defect area is observed by HE staining, and the repair of bone defect is observed by Mo-target radiography. Histological examination results show that, at the 4th week after surgery, there was still a small amount of sECMH in defect area. This indicates that, the duration of sECMH absorption and degradation in femoral neck defect area is about 4 weeks. During this period sECMH is gradually absorbed and degraded, with the growth of surrounding tissue, and no cell growth at the center of sECMH is visible (**Figure 2A** and **2C**).

At the 2nd postoperative week, HE staining results show that, there is fibrous tissue hyperplasia in defect area, with no difference between experimental side and control side. At the 4th week, there is uniformly distributed osteoid in fibrous tissue in defect area in experimental side, with abundant blood supply. In control side, there is no vascular structure in the center of defect area. The osteoid in fibrous tissue is gradually reduced from outside to inside. The blood supply is an essential condition for bone tissue regeneration. In the defect area of experimental side, the abundant blood supply in fibrous tissue has provided sufficient raw material for bone tissue regeneration. So the fibrous tissue in all defect area is synchronously transformed into bone tissue. In control side, with the decreasing of blood supply in fibrous tissue from outside to inside, the amount of new osteoid is also reduced from outside to inside. In the center of defect area, only the fibrous tissue is visible, with no appearance of osteoid. This phenomenon is also reflected by Mo-target radiography at the 4th postoperative week. The relatively high-density image appears in defect area of experimental side, with relatively low-density image in control side.

At the 8th postoperative week, as shown in gross observation results, the marrow cavity is formed in the experimental side, which is also confirmed by HE staining. The marrow cavity is filled with haemocytes, adipocytes and trabecular structure along the force line. The defect orifice is closed with new cortical bone smoothly extending from surrounding normal bone. The osteoblasts are hypertrophic, and in functional status. In control side, the marrow cavity was filled with a large amount of bone callus. HE staining results show that, there is a lot amount of irregularly arranged trabecular structure, with external attachment of a small amount of fibrous tissue, but it is still represented as a cupped defect. This can also be observed in Mo-target radiography. In experimental side, there was a gradual extension from normal cortex area to defect area, with unclear boundary between them. In control side, the high-density image appears at defect edge. The regeneration and reconstruction of bone are performed at the same time. The continued strengthening of structure on stress line and absorption of structure in non-stress-bearing area constitute the repair process of bone tissue. As shown in HE staining, at the 8th postoperative week, the defect part is almost recovered to normal morphology. Results of this study are in accordance with the conclusions of Matsubara et al. [19] Gong et al. [20] that, using the HGF gene transfection method, HGF can be expressed and released in bone defect area, thus promoting the bone regeneration.

Conclusion

As a new drug delivery system, sECMH containing HGF has good application prospect in bone tissue repair.

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Disclosure of conflict of interest

None.

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References

[1] Parsons B and Strauss E. Surgical management of chronic osteomyelitis. Am J Surg 2004; 188: 57-66.

[2] Khosla VM. Reconstruction of a mandibular defect with a homogeneous bone graft: report of case. J Can Dent Assoc (Tor) 1971; 37: 35-37.

[3] Davis WH, Delo RI, Weiner JR, Terry B. Transoral rib grafting for mandibular alveolar atrophy--a progress report. Trans Int Conf Oral Surg 1973; 4: 206-209.

[4] Joshi A, Kostakis GC. An investigation of postoperative morbidity following iliac crest graft harvesting. Br Dent J 2004; 196: 167-171.

[5] Cancedda R, Giannoni P, Mastrogiacomo M. A tissue engineering approach to bone repair in large animal models and in clinical practice. Biomaterials 2007; 28: 4240-4250.

[6] Imai Y, Terai H, Nomura-Furuwatari C, Mizuno S, Matsumoto K, Nakamura T, Takaoka K. Hepatocyte growth factor contributes to fracture repair by upregulating the expression of BMP receptors. J Bone Miner Res 2005; 20: 1723-1730.

[7] Prestwich GD. Simplifying the extracellular matrix for 3-D cell culture and tissue engineering: a pragmatic approach. J Cell Biochem 2007; 101: 1370-1383.

[8] Shu XZ, Ahmad S, Liu Y, Prestwich GD. Synthesis and evaluation of injectable, in situ crosslinkable synthetic extracellular matrices for tissue engineering. J Biomed Mater Res A 2006; 79: 902-912.

[9] Elia R, Fuegy PW, VanDelden A, Firpo MA, Prestwich GD, Peattie RA. Stimulation of in vivo angiogenesis by in situ crosslinked, dual growth factor-loaded, glycosaminoglycan hydrogels. Biomaterials 2010; 31: 4630-4638.

[10] Liu Y, Cai S, Shu XZ, Shelby J, Prestwich GD. Release of basic fibroblast growth factor from a crosslinked glycosaminoglycan hydrogel promotes wound healing. Wound Repair Regen 2007; 15: 245-251.

[11] Riley CM, Fuegy PW, Firpo MA, Shu XZ, Prestwich GD, Peattie RA. Stimulation of in vivo angiogenesis using dual growth factor-loaded crosslinked glycosaminoglycan hydrogels. Biomaterials 2006; 27: 5935-5943.

[12] Zhao J, Zhang N, Prestwich GD, Wen X. Recruitment of endogenous stem cells for tissue repair. Macromol Biosci 2008; 8: 836-842.

[13] Qin LX, Tang ZY. The prognostic molecular markers in hepatocellular carcinoma. World J Gastroenterol 2002; 8: 385-392.

[14] Fuller K, Owens J, Chambers TJ. The effect of hepatocyte growth factor on the behavior of osteoclasts. Biochem Biophys Res Commun 1995; 212: 334-340.

[15] Inaba M, Koyama H, Hino M, Okuno S, Terada M, Nishizawa Y, Nishino T, Morii H. Regulation of release of hepatocyte growth factor from human promyelocytic leukemia cells, HL-60, by1,25-dihydroxyvitaminD3,12-O-tetradecanoylphorbol 13-acetate, and dibutyryl cyclic adenosine monophosphate. Blood 1993; 82: 53-59.

[16] Takai K, Hara J, Matsumoto K Matsumoto K, Hosoi G, Osugi Y, Tawa A, Okada S, Nakamura T. Hepatocyte growth factor is constitutively produced by human bone marrow stromal cells and indirectly promotes hematopoiesis. Blood 1997; 89: 1560-1565.

[17] Liu JM, Ning G, Chen JL. Osteeporotie fractures in Asia: risk factorsand strategies for prevention. J Bone Miner Metab 2007; 25: 1-5.

[18] Zheng Shu X, Liu Y, Palumbo FS, Luo Y, Prestwich GD. In situ crosslinkable hyaluronan hydrogels for tissue engineering. Biomaterials 2004; 25: 1339-1348.

[19] Matsubara H, Tsuchiya H, Watanabe K, Takeuchi A, Tomita K. Percutaneous nonviral delivery of hepatocyte growth factor in an osteotomy gap promotes bone repair in rabbits. Clin Orthop Relat Res 2008; 466: 2962-2972.

[20] Gong EN, Shang FY, Guo ZK, Lou X. Transplantation of hepatocyte growth factor gene-modified mesenchymal stem cells in the treatment of femoral head necrosis in rabbits. J Clin Rehabil Tissue Engineering Res 2009; 13: 5411-5415.