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Histological analysis of bone repair in rat femur *via* nanostructured merwinite granules

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Abstract

There is a conspicuous need for *in vivo* evaluation of biomaterials prior to their application as bone graft or tissue engineering scaffold. Merwanite and HA *in vivo* application as bone filler would be studied in this study. Femoral injury was induced in male rats and early and late degrees in bone repair were evaluated. A histomorphological analysis of the bioceramics implants in rat femoral defect models suggested that both in early and late stage of bone repair, merwinite is more effective in promoting osteogenesis in comparison with HA and in late stage, the rate of new bone formation was faster in merwinite-filled-bone-defect than in HA models. The control groups showed limited osteogenesis. These results suggested that merwinite might be a potential and attractive bioceramic for bone replacement.

Keywords: Bioceramic; Merwinite; *In vivo*; Histological analysis

1. Introduction

Previous studies have shown that calcium, magnesium and Si-containing bioceramic possessed good bioactivity and can be used for biomedical purposes [1–4]. They are highly bioactive and *in vitro* adhesion and proliferation of osteoblasts on their surface is enhanced. *In vitro* and *in vivo* evaluation of diopside confirmed that it possesses apatite formation ability in SBF and could bond with bone when implanted in rabbits [5,6]. Bredigite showed higher cell proliferation rate and differentiation level in comparison with β-Tricalcium phosphate (β-TCP) [7]. *In vivo* evaluation of akermanite showed that the rate of bone formation was faster when compared with β-TCP [8]. It is shown that, diopside has bone bonding ability when it is implanted in rabbits [9]. Recently, merwinite [$Ca_3Mg(SiO_4)_2$] has received more attention because of enhancing more cell proliferation and better mechanical properties [10]. In our previous study, osteoblasts have displayed better activities of proliferation on merwinite than HA [11].

Calcium phosphate bioceramics, especially HA, due to their similarities to mineralized composition of hard tissues, broadly use as bone implants and grafts [12–16]. However, there are number of limitations in application of HA (such as low compressive strength and fracture toughness) which restrict its wider applications in bone repairs [17].

In the present study, a rat model with surgically induced femoral condyle injury was used to investigate the *in vivo* osteogenic ability of merwinite ceramic implants, in comparison with HA ceramics and empty defect.

2. Materials and methods

2.1. Materials and experiments

In our previous study, nanocrystaline merwinite powders were synthesized using the sol–gel method [3].

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The physical and *in vitro* evaluation of prepared nanostructured merwinite have been described in details previously [3,11]. Pure hydroxyapatite powder was used as received (Merck, Art. no. 2196). The merwinite sample was analyzed by X-ray diffraction (XRD-Philips PW 3710 diffractometer) and Transmission Electron Microscopy (TEM-Philips EM 208S). 24 Male Lewis rats 3–4 months old, with average weight (260 ± 20 g) were divided into 3 groups 1 week before experiments. The animals were divided into two test groups of A and B, with group A referring to the animal treated by merwinite and B with HA. Each test group comprises of 8 rats. 8 rats with surgically-induced bone defect was left without any implanted material as control group, They were housed under optimal housing and feeding conditions. All experimental and control groups were handled in accordance with institutional and national guideline for the care and use of laboratory animals.

Following applying anesthesia for each animal, the surgery was done and the granules of merwinite (in group A) and HA (in group B) were implanted bilaterally into the experimentally induced femoral bone defects (0.3 cm in diameter and 0.2 cm in depth). The washing was applied using sterile saline solution and then the subcutaneous tissues and skin were sutured. Identical surgical operation was performed on control group rats, however, the induced-bone defect was left unfilled. After the operation, each rat was placed in special cage.

2.2. Histological and morphological studies

To evaluate the early and late effects of two different kinds of biomaterials on bone formation, all animals were sacrificed by deep anesthesia 2 and 8 weeks after operations. The implanted materials and surrounding tissue were removed and the sites of implantations were cut for tissue processing. All femoral bones were fixed in 4% paraformaldehyde. Decalcification was done by 5% nitric acid, and dehydration was done by graded series of alcohols from 50% to 100%. Clearing and impregnation of specimens were done by Xylene and melted paraffin, respectively. Each bone was cut into two pieces and then placed in two sides; including vertical and horizontal paraffin blocks. A series of 5 μm thick sections at various levels (25–30 μm interval) were obtained from each tissue block and then stained with hematoxylin–eosin (H&E) and Masson's trichrome methods according to standard protocols. The slides were observed by light microscope (BX51 Olympus, Japan) at different magnifications.

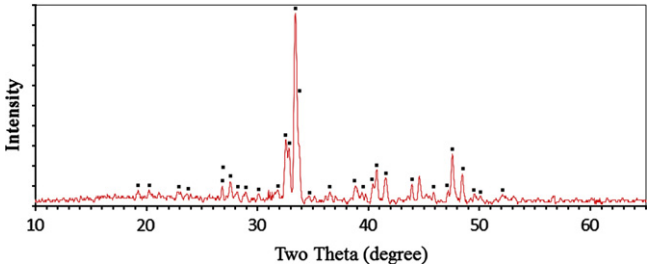

Fig. 1. XRD pattern of merwinite powder.

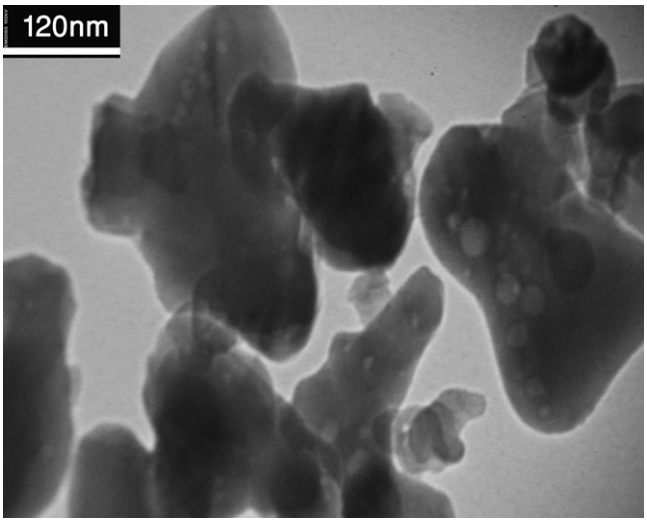

Fig. 2. TEM image from synthesized merwinite particles.

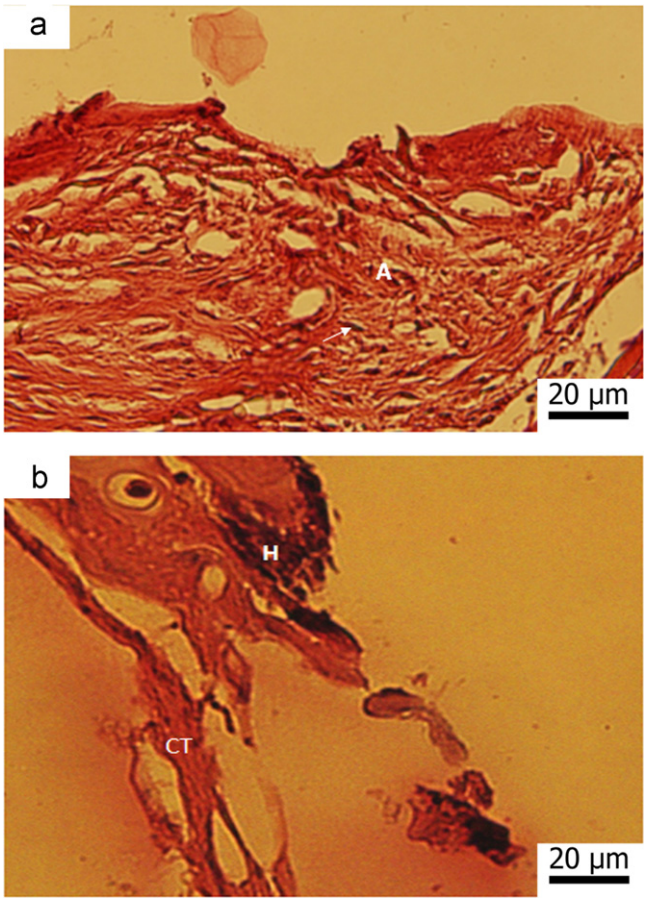

Fig. 3. bone injury repair in HA-implanted animals (group B) after 2 weeks: (a) Invasion of connective tissue elements into the site of injury for repair, but at this time there is no sign of bone formation. The arrow shows a fibroblast. (b) H indicates the HA in site of injury. CT shows the connective tissue. There is no sign of osteogenesis.

3. Results and discussion

The XRD and TEM analysis of merwinite powders showed that phase was pure and crystallite size was below 100 nm (Figs. 1 and 2).

In our study, no bone formation was observed in HA-implanted animals (group B) after 2 weeks, where most of the defect sites were filled with loose and fibrous connective tissues (Fig. 3a and b). While, invasion of connective tissue elements and formation of small bone islands were observed in merwinite-implanted animals (group A) at this time (Fig. 4a). Immature trabecula of bone, residual of merwinite, connective tissue elements and osteoclasts were observed in Fig. 4b. Immature bone and connective tissue were seen on the left and right side of image, respectively (Fig. 4c). Synthesis of collagen fibers by osteoblasts in immature trabecula of bone were observed by Masson's trichrome method (Fig. 4d).

Eight weeks after implantation with HA, the osteocytes in lacuna, the new Haversian system with wide canal and osteoblasts were observed (Fig. 5a and b). Comparing with tissue examined 2 weeks after operation, more cell differentiation to osteoblasts was observed after 8 weeks. Several irregular trabecula of newly formed bones were observed in many parts of specimens. On the other hand, in some areas, maturating bone trabecula was seen and forming Haversian systems was started. In other word, in newly formed bones, many osteocytes in lacuna and osteoblasts could be seen. More bone callus formation was observed in comparison with 2 weeks and HA for 8 weeks evaluations (Fig. 6a. Fig. 6b shows both lamellar and immature bone near to each other. It is noteworthy that the tissue shows a transition from immature to mature bone tissue after 8 weeks. Fig. 6c also shows the signs of bone remodeling and the persistence of lamellar bone. Formation of Haversian system in newly formed bone was observed, as well Fig. 6d shows that synthesis of collagen bundles was completed, as bone lamella and Haversian system formation are delineated using trichrome masson staining. In control group, empty bone defects showed few new bone formation, limited to the surrounding of the defect (Fig. 7a–c). Connective tissue was observed which suggests delay in bone formation at both 2 and 8 weeks following injury. In sum, bone regeneration was undergoing in control animals but with slower pace and smaller areas than other groups.

In comparison with HA-implanted group, new bone formation and material degradation were much more evident in merwinite-implanted rats after both 2 and 8 weeks. Furthermore, after 8 weeks of implantation, the newly formed bone tissue penetrated into the center of merwinite implants associated with much more material degradation (Figs. 3 and 5a).

As previous studies have shown, Si and Mg have been found in the dissolution of merwinite ceramics [11]. A significant characteristic of a bioactive material is its ability to bond with living bone through the formation of an apatite interface layer on their surface. We showed that

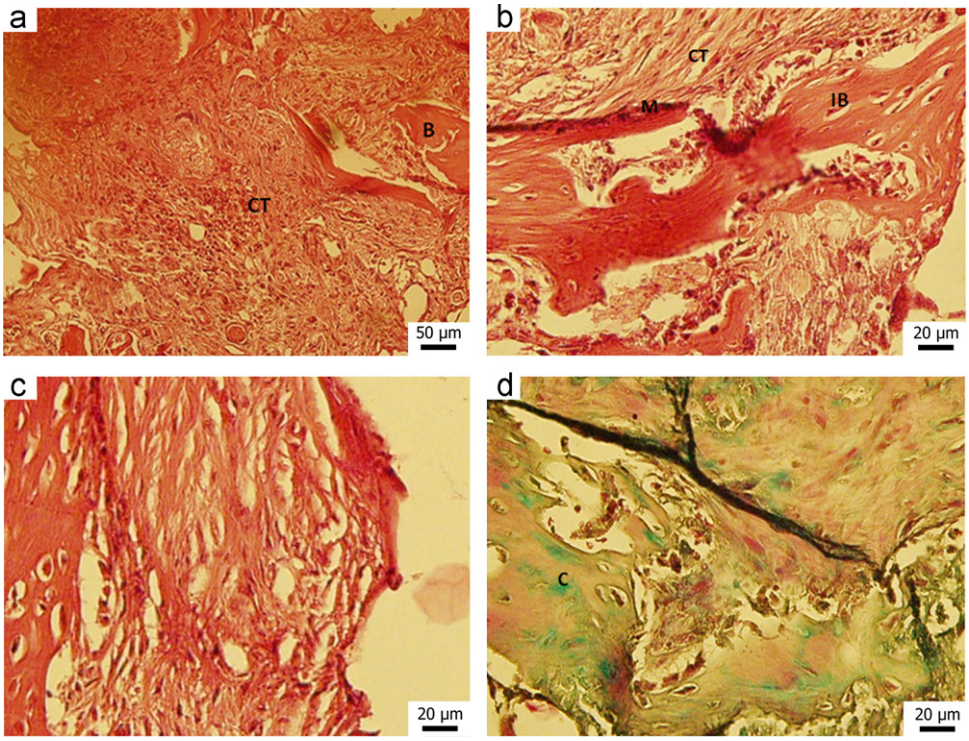

Fig. 4. Bone injury repair in merwinite-implanted animals (group A) after 2 weeks: (a) Invasion of connective tissue elements (CT) and formation of small bone lslands (B). (b) Immature trabecula of bone (IB). Merwinite (*M*), connective tissue (CT). (c) Immature bone on the left side and connective tissue on the right side of image. (d) Collagen (C) fibers in immature trabecula of bone (Massone's trichrome staining).

the merwinite possessed apatite-formation ability in SBF, showing their proper bioactivity. The potential mechanism for this apatite-formation ability on merwinite ceramics could be through the selective adsorption of Ca^{2+} to Si–OH and subsequent adsorption of phosphate ions to form apatite [3,11].

Investigation of the *in vivo* osteogenetic ability of any materials for bone tissue engineering is needed. In addition, the degradation rate of scaffolds must be coupled appropriately with the rate of new bone tissue formation. The results of the present study suggested that, at the late stage of implantation (after 8 weeks), the newly formed

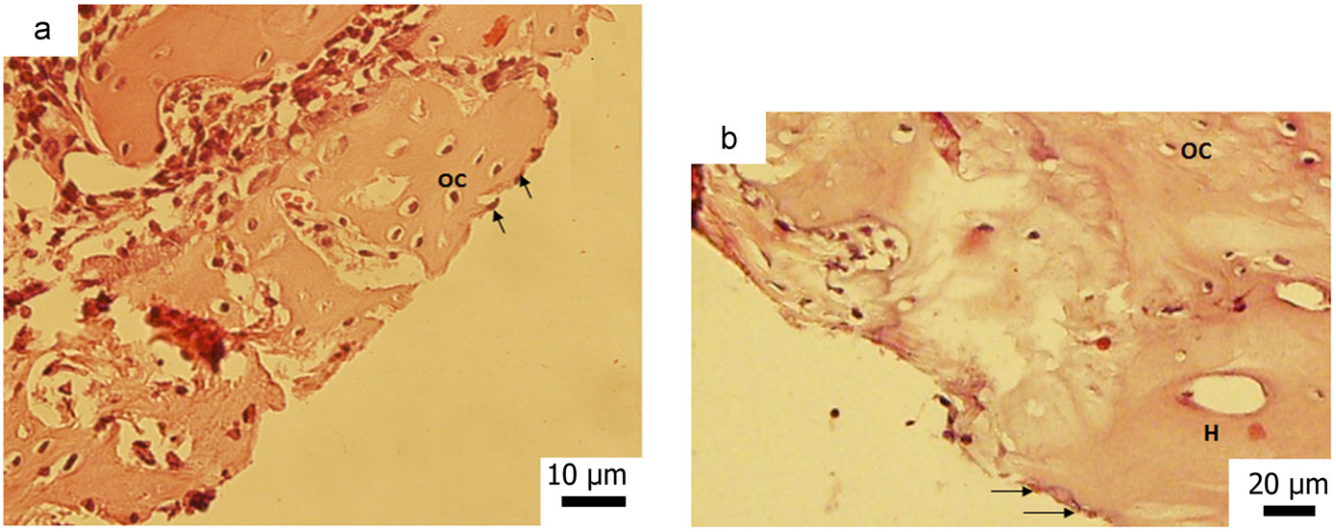

Fig. 5. Osterogenesis in HA group after 8 weeks. (a) Osteocyte in lacuna (OC), New Haversian system (H) with wide canal. Osteoblasts (arrows). (b) Newly formed bone with osteocytes (OC) in lacuna and osteoblasts (arrows).

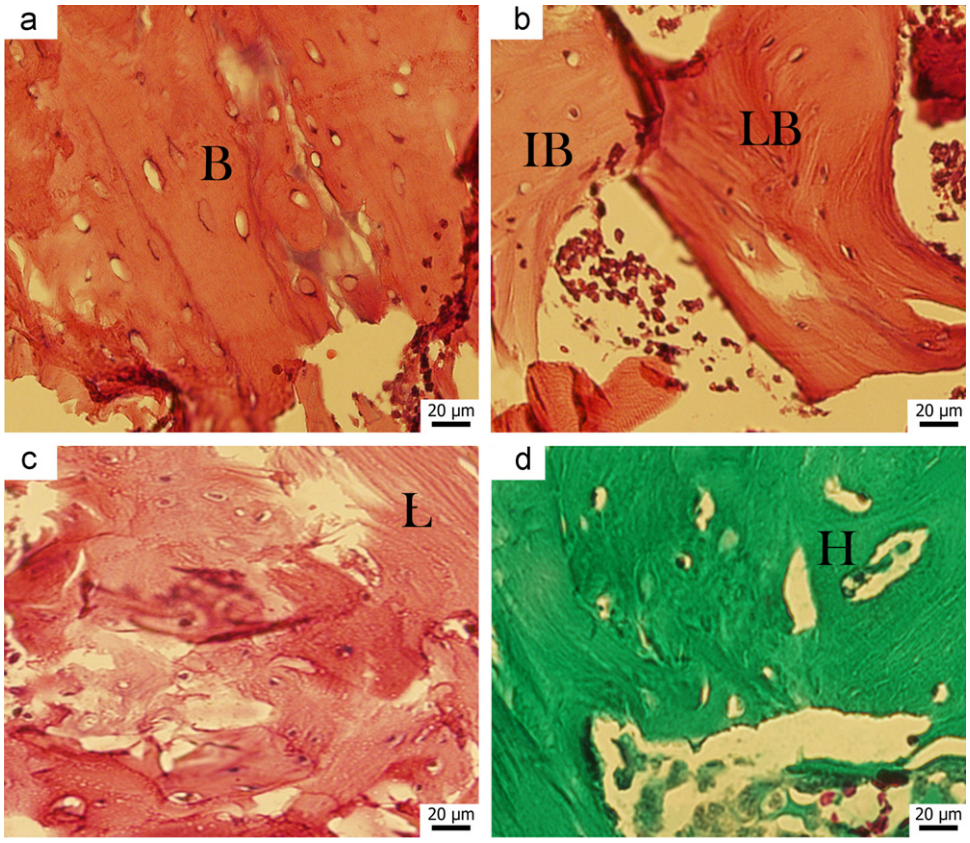

Fig. 6. Bone formation in merwinite-implanted bones after 8 weeks: (a) Bone formation (B) in wide range. (b) Lamellar bone (LB) and immature bone (IB). (c) The signs of bone remodeling and the persistence of lamellar bone (*L*). (d) Formation of Haversian system (H) in newly formed bone (Massone's trichrome staining).

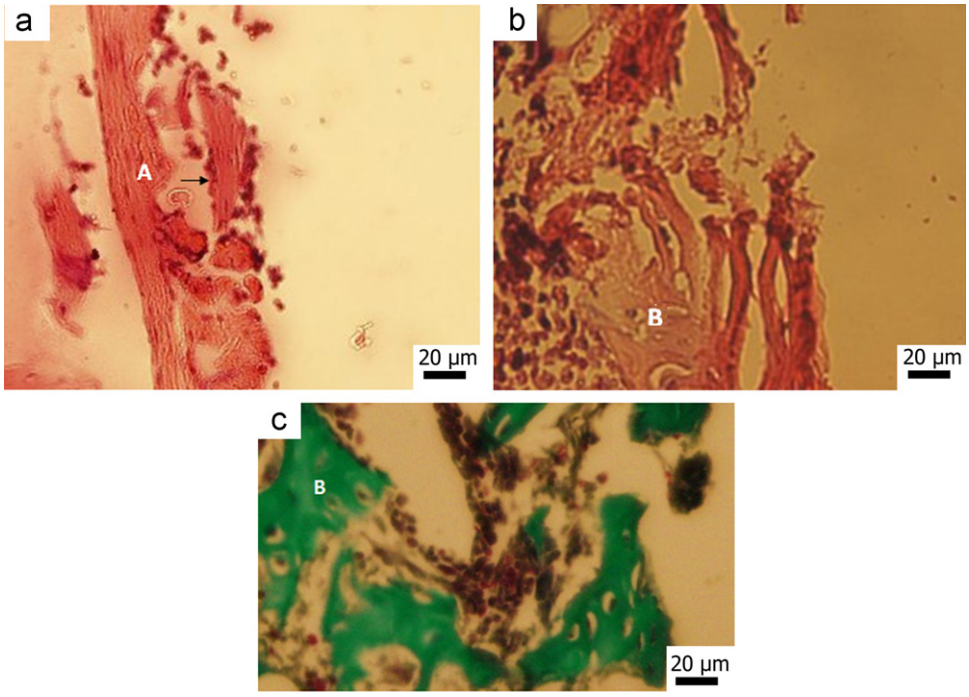

Fig. 7. Bone repair in control group animals after 8 weeks. (a) Newly formed immature bone trabecula with osteocytes. The arrow shows active osteoblasts, (b) limited immature bone (B) formation in the site of injury, (c) Immature bone (B) formation (Massone's trichrome staining).

bone tissue penetrated into the center of the merwinite, while in the case of HA implants, the newly formed tissue was mainly on the edge of the ceramics. This behavior suggesting that the biodegradation and biocompatibility of merwinite is more proper for the new bone formation *in vivo* than HA. This study suggests that the implanted merwinite in bone defects dissolve to release diffusible factors that induce a new bone formation. It is believed that these factors release into the bone marrow and have a positive chemotactic action on osteogenic stromal cells. This led to activating and differentiating of osteogenic cells from bone marrow to form osteoblasts. These activated cells synthesized abundant extracellular matrix [18–26]. In our observations, the functional new bone trabeculae that were formed in close contact with merwinite, were covered with osteoid and lined with osteoblasts, have indicated the continuous bone formation. It should be noted that the bone is constantly remodeled by the actions of osteoblasts and osteoclasts. Ideal biomaterial should support the activity of both osteoblasts and osteoclasts to establish normal bone remodeling at the interface. However, in present study, we saw that the newly formed bone was remodeled and developed into mature and organized lamellar bone.

On the other hand, we saw the osteogenesis was wider and faster in merwinite-implanted bones when compared with HA-implanted and control specimens. So, it is suggested that the biodegradation and biocompatibility of merwinite for *in vivo* new bone formation is more proper than HA.

4. Conclusion

In vivo implantation of two granule bioceramics in rat femoral defect model demonstrated that the rate of new bone formation is faster and wider in merwinite than in both HA and untreated injury after 8 weeks of the implantation. Furthermore, the degradation of merwinite ceramics is also faster than HA ceramics. *In vivo* results suggest that the merwinite ceramics has good bioactivity and thereby can stimulate more proliferation and differentiation of bone progenitor cells than HA. Also it enhances bone regeneration because of its superior degradation rate and proper biocompatibility.

Therefore, merwinite might be used as a potential and attractive bioceramic for bone regeneration and bone tissue engineering applications.

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