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Enhanced osteogenic healing process of rat tooth sockets using a novel simvastatin-loaded injectable microsphere-hydrogel system

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ABSTRACT

Purpose: To evaluate the effects of simvastatin in a new injectable microsphere hydrogel system on bone healing process of tooth sockets.
Materials and methods: Simvastatin was loaded in poly (lactic-co-glycolic acid) (PLGA) microspheres using an emulsion process, and the drug-loaded PLGA microspheres were further entrapped in a gelatin hydrogel to form an injectable microsphere-hydrogel system. Simvastatin-free hydrogel and blank microspheres hydrogel were used as controls. A rat tooth extraction socket model was generated, and the simvastatin-loaded microsphere-hydrogel composite was injected in the defect area of a tooth socket. At 1, 2, 5, and 8 weeks after the surgery, all the animals were sacrificed and the mandibles were harvested. The samples were examined using X-ray, hematoxylin and eosin staining, and histological evaluations.
Results: Five weeks after the surgery, significantly more bone tissue was formed in the simvastatin-loaded hydrogel group than in the simvastatin-free hydrogel group and the blank microspheres hydrogel group as control ($p < 0.05$).
Conclusion: The injectable simvastatin-loaded microsphere hydrogel promoted new bone formation in the tooth extraction socket after 5 weeks, and has a promising potential for bone repair and regeneration.

1. Introduction

Resorption of alveolar bone that is termed “residual ridge resorption” (RRR) often occurs to different degrees after tooth extraction. The RRR is chronic, progressive, irreversible and cumulative, and often results in many aesthetic and functional problems in edentulous patients. Therefore, it is pivotal to reduce or to prevent RRR after the tooth extraction. Theoretically, grafting of autologous bone into extraction socket can promote defect healing and reduce RRR. However, this treatment not only creates a secondary injury, but also is difficult to fit the irregular shape of an extracted socket (Checchi et al., 2011; Kubo et al., 2011). Some synthetic biomaterials, such as hydroxyapatite and tricalcium phosphate, have been implanted into extraction sockets for alveolar bone regeneration (Checchi et al., 2011). However, these materials are not osteoinductive, and the degradation process does not match the rate of new bone formation. Therefore, it is necessary to develop new osteoinductive biomaterials for bone regeneration for RRR treatment.

Mundy et al. first reported that statins induced bone morphological protein 2 (BMP-2) gene expression in osteoblasts and bone marrow cells, indicating that statins play a crucial role in modulating bone formation (Mundy et al., 1999). A previous study demonstrated that the resorption of alveolar ridge after tooth extraction was effectively prevented by local application of simvastatin (Wu et al., 2008). Another study showed that simvastatin inhibited the resorption of periodontal tissues and promoted bone regeneration (de Mones et al., 2015). These results suggested that simvastatin has a positive effect on bone repair of extracted sockets.

While simvastatin has therapeutic effects, its bioavailability of oral administration is lower than 5% after hepatic metabolism (Kato, 2008), and a much smaller amount of the simvastatin goes into the targeted bone defective area. On the other hand, a high amount of simvastatin may cause serious side effects, including muscle and liver toxicity (Norata et al., 2014; Gee et al., 2015). A

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number of biomaterials, such as collagen sponges (Wong and Rabie, 2005; Calixto et al., 2011; Ricky and Wong, 2005), gelatin (Tanigo et al., 2010), poly (lactic-co-glycolic acid) (PLGA) (Wu et al., 2008; Tanigo et al., 2010), and methyl cellulose (Lee et al., 2008; Pradeep et al., 2015) were used as simvastatin delivery carriers for bone formation. These scaffolding materials were not injectable and did not fit the irregular extraction sockets, leading to unsatisfactory healing efficacy. In this study, we developed a unique injectable simvastatin-loaded microsphere hydrogel system for an enhanced osteogenic healing process of tooth sockets. Using a rat incisor extraction socket model, we evaluated the effect of our injectable system on osteogenic healing in extracted sockets (Fig. 1).

2. Materials and methods

2.1. Preparation and characterization of simvastatin-loaded PLGA microspheres

One gram of PLGA (molar ratio of PLA/PGA was 1:1) was dissolved in 10 ml of dichloromethane. A 0.01-g quantity of simvastatin (Merck, USA) was dispersed into the PLGA solution. The mixture was added to 10 ml of 2% poly (vinyl alcohol) (PVA) solution and was continuously stirred at 500 rpm for 24 h. The white precipitation was collected by centrifugation at speed of 3000 rpm and washed with deionized water and alcohol. The obtained microspheres were dried and characterized by scanning electron microscopy (SEM) (JSM-840, JEOL Ltd., Japan). The microspheres were sterilized with gamma radiation (Co60) before using them in cell culture experiments.

2.2. Preparation of gelatin hydrogel containing microspheres loaded with simvastatin

Simvastatin-loaded PLGA microspheres (0.1 g, including 0.01 g simvastatin), gelatin (0.2 g), glycerin (1 ml), and deionized water (2 ml) were mixed using ultrasonic agitation at room temperature to form a homogeneous hydrogel, which was subsequently loaded into a sealed syringe. Simvastatin (0.01 g) and PLGA microspheres (0.1 g) alone were used as simvastatin-free hydrogel and blank microspheres hydrogel, respectively. All hydrogels were sterilized using cobalt-60 irradiation and preserved at 4 °C for later use. The loading capacity (LC) and encapsulation efficiency (EE) were calculated as follows:

LC = (the amount of simvastatin within microspheres/total weight of microspheres) × 100%

EE = (the amount of simvastatin within microspheres/total simvastatin) × 100%

2.3. Release of simvastatin from gelatin hydrogels

Simvastatin released from gelatin hydrogels and from microsphere hydrogel systems were examined in vitro. Each sample was placed in a glass vial containing 20 ml of phosphate-buffered saline solution (PBS, pH 7.0) used as a releasing medium. The sealed vials were placed in a water bath at 37 °C and were shaken at a speed of 15 rpm. At 1, 3, 7, 14, 21 and 28 days, the supernatants were collected from the vials and replenished with fresh PBS. The optical density (A) of simvastatin was obtained from ultraviolet spectroscopy at 238 nm (Lambda800). According to a standard curve equation $A = 0.07856C + 0.00124$ ($r = 0.9993$) (Wu et al., 2008), the simvastatin concentration (C) of each sample was calculated, and the average value was reported.

2.4. In vitro cell viability

MC3T3 cells (CRL-2593; ATCC, Manasas, VA) were cultivated in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum, 0.01% ascorbic acid and 100 U/ml penicillin/streptomycin at 37 °C in a 5% CO_2 humidified incubator. The culture medium was replenished every other day. For subculture, the cell monolayer was rinsed with PBS and detached by incubation with trypsin–ethylenediaminetetraacetic acid (EDTA) (0.25 wt %) for 5 min at 37 °C. Cell viability was quantitatively assessed with a tetrazolium compound, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxy methoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS, Sigma) at 24 h. MTS, in the presence of phenazinemethosulfate (PMS), yields a water-soluble formazan product that has a maximum absorbance at 490 nm in PBS. For the cultured cells, the amount of formed colored product was proportional to the number of viable cells and the incubation time with MTS/PMS. For cytotoxicity evaluation, aliquots of 5×10^3 cells per well were seeded in a 96-well cell culture plate and cultured at 37 °C in a 5% CO_2 humidified incubator. Simvastatin-loaded microspheres (30 μg/ml) and blank PLGA microspheres (30 μg/ml) were added in each well and cultured at 37 °C in a 5% CO_2 humidified incubator as controls.

After 24 h of cell culture, 10 μl of the MTS solution (5 mg/ml in PBS) was added to each well and incubated for 4 h. The optical density (OD) of the resultant water-soluble formazan in the solution was measured. Five samples in each group were collected and the average was reported.

2.5. Animal experiments

A total of 36 Wistar male rats (Jilin University Experimental Animal Center, weight 180 g ± 5 g) were used for animal studies. All animals ate and drank freely at 22 °C under 40% humidity following the reversal of the daylight (12:12 h) cycles. The rats were randomly divided into three groups (simvastatin-loaded microspheres

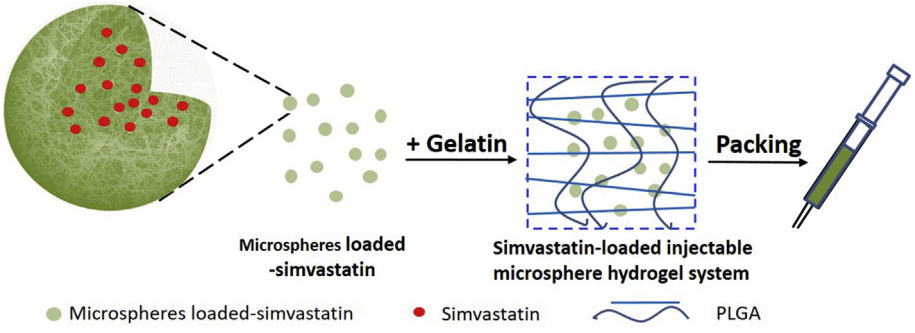


Fig. 1. Schematic illustration of the preparation of simvastatin (Sim)-loaded microspheres hydrogels and its application into rat extraction socket.

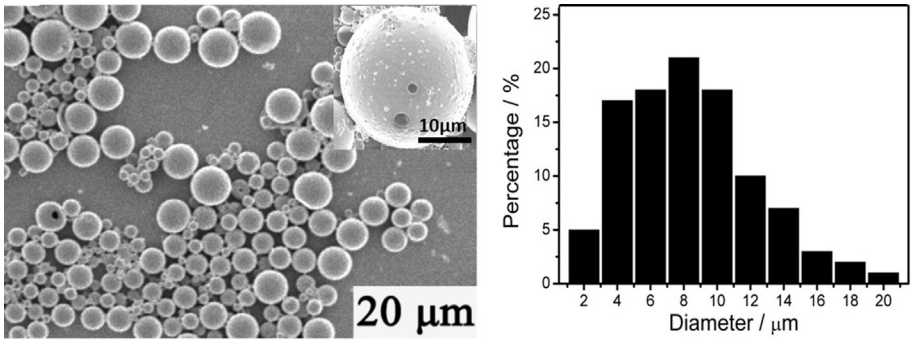
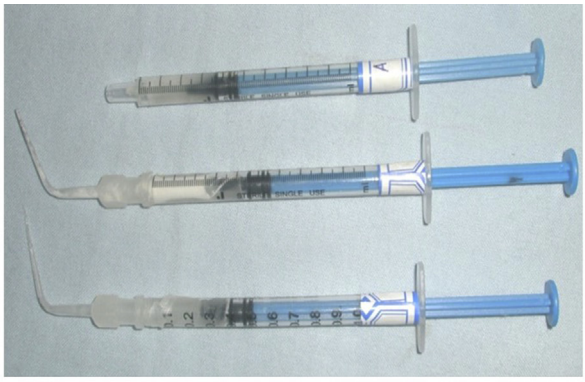


Fig. 2. Characterization of simvastatin (Sim)-loaded PLGA microspheres (MS). (A) SEM image of the Sim-loaded MS, overview. The inserted high magnification image is the SEM image of a typical Sim-loaded MS, showing that the MS is regularly spherical and contains micro-pores. (B) Size distribution of the Sim-loaded MS.



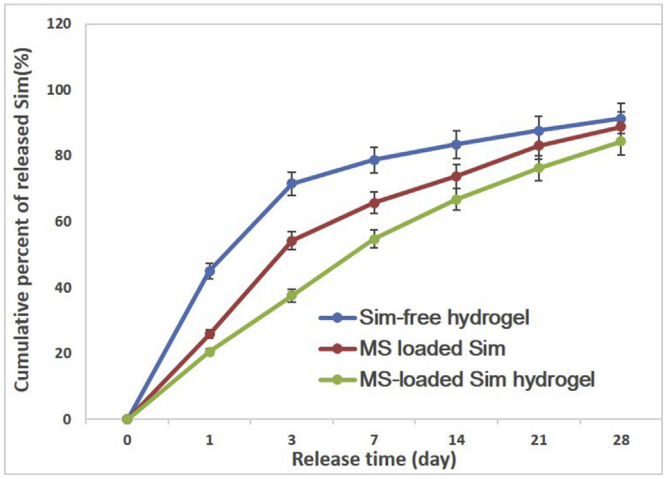


Fig. 3. (A) Photographs of simvastatin (Sim)-free and Sim-loaded microspheres (MS) hydrogels. (B) Cumulative release profiles of simvastatin from simvastatin-free hydrogels, Sim-loaded MS, and Sim-loaded MS hydrogels.

hydrogel, simvastatin-free hydrogel, and blank microspheres hydrogel, n = 12). Removal of the right mandibular central incisor was performed under general anesthesia using an injection of 0.3 ml/kg SU-MIAN-XIN (Academy of Military Medical Science, Changchun Institute of Military vet). The tooth socket was filled with the selected biomaterial in each group and the opening covered with periodontal dressing agents (Hager Werken, Germany). The rats were fed a soft diet for 2 days and were given ampicillin sodium for 3 consecutive days (60 mg/kg). At 1, 2, 5 and 8 weeks after the surgical procedure, 3 animals in each group were randomly selected and sacrificed. The sacrificed animals were then

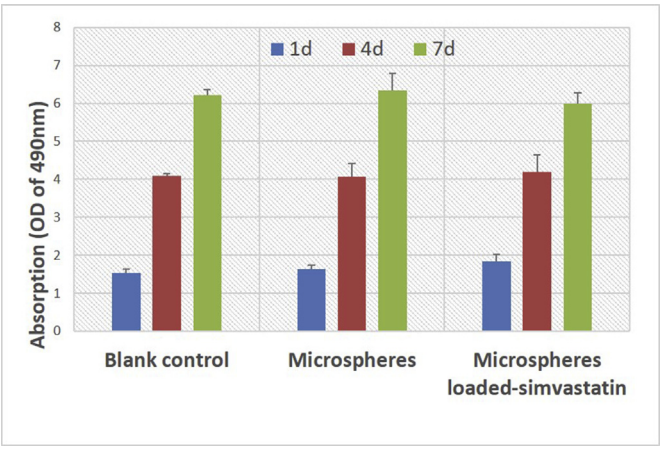


Fig. 4. Biocompatibility assay of the microspheres (MS) and simvastatin (Sim)-loaded MS. The blank control was used as a negative control. There were no significant difference of cell numbers among the three groups at days 1, 4, and 7 ($p > 0.05$).

perfused constantly by a MINI-type infusion pump for fixation using 4% paraformaldehyde. After complete fixation, mandibles were separated and immersed in the 4% paraformaldehyde for 24 h. The animal surgical procedure was approved by the University Committee on Use and Care of Animals (UCUCA) of Jilin University College of Dentistry (Protocol# 2016-0214).

2.6. Soft X-ray observation of mandible

The mandible samples removed throughout soft tissues underwent soft X-ray with a molybdenum target camera (Sitto, IMS Company). The parameters of projection were 29 kv, 8–11 mAs, and the socket areas of mandibles (Fig. 5B) on photographic images were captured by an EPSON3490 scanner (parameter settings: 48 full-color, 1200 dpi), respectively. The image analysis system IPP6.0 (Media Cybernetics, USA) were applied to compare and analyze the gray range of images of the incisor tooth extraction socket in each group, and to evaluate the bone mineral density in the socket of the extracted tooth.

2.7. Histology

The mandible samples were decalcified in 10% EDTA, and the mandibular tissue blocks including the lower first molar 3.0 mm in thickness underneath the lower first molar (as shown in Fig. 5A) were cut as samples. The samples were dehydrated, wax infiltrated, embedded and sectioned with a thickness of 5 μm. After

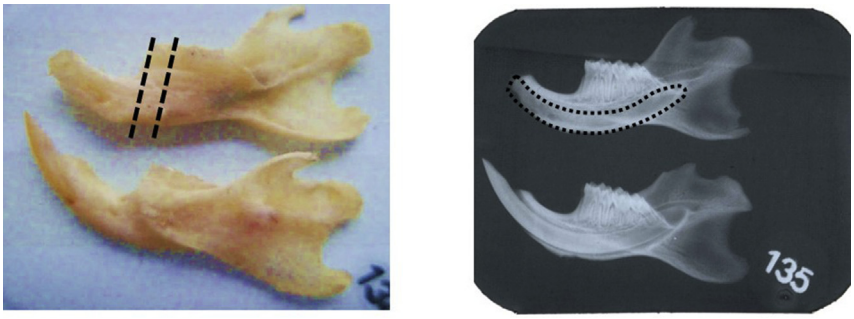


Fig. 5. Rat mandibles (A) and their soft X-ray photographs (B). The uppers are mandibles with extracted incisors, and the lowers are mandibles without extracted incisors. In (A), the bone tissue blocks with the lower first molar (between two slashes) were harvested for tissue observation. In (B), the area circled by the dotted line represents the incisor's socket and was scanned for estimating the bone density of the socket.

hematoxylin and eosin staining, the sections were observed under an optical microscope.

2.8. Statistical analysis

All the data were analyzed with SPSS software (version 13.0; SPSS, Chicago, IL). The results were expressed as mean ± standard deviation. The difference among three groups was compared by one-way analysis of variance. If there is a difference among the three groups, a Tukey post hoc test was calculated. The significance level was set at $P < 0.05$.

3. Results

3.1. Characterization of simvastatin-loaded PLGA microspheres

Fig. 2 shows the simvastatin-loaded PLGA microspheres, which possessed typically spherical shape. The microspheres had rough surfaces and an average size distribution of 8.0 μm (Fig. 2B). The simvastatin-loaded microspheres had a loading capacity of 10.2% and an encapsulation efficiency of 39.0%.

3.2. In vitro simvastatin release

The simvastatin-loaded microsphere gelatin hydrogels were conveniently transferred into syringes without any sediment at room temperature (Fig. 3A). These drug-loaded hydrogels had excellent flow property at 37 °C, indicating they could be injected into the desired site, which is beneficial for clinical application. While both simvastatin-loaded microspheres and hydrogel -containing simvastatin-loaded microspheres released simvastatin for 4 weeks, the hydrogel containing simvastatin-loaded microspheres had a more stable release profile than that of the simvastatin-loaded microspheres. As a comparison, hydrogel that was simvastatin-free rapidly released simvastatin in a shorter period (Fig. 3B).

3.3. Cell viability

Fig. 4 shows the cell viability results after the cells were exposed to microspheres (with or without simvastatin) and the blank control and incubated for 1, 4, and 7 days. Overall, the cell numbers in all three groups started to increase after day 1, and there were no statistically significant difference among the three groups, indicating that the simvastatin-loaded microspheres hydrogel did not hinder cell replication and thus could be considered biocompatible.

3.4. Bone density examination of mandibles

Bone density of all mandible samples was determined and then the average gradation of incisive fossa in each film was analyzed by IPP6.0 software. One week after surgery, bone mineral density of the extraction socket of the two simvastatin-containing groups was higher than that of the blank MS hydrogel group ($p < 0.05$), but between the two simvastatin-containing groups there was no significant difference ($p > 0.05$). At the second week, bone density of the simvastatin-free hydrogel group was significantly higher than the simvastatin-loaded microsphere hydrogel group and the blank hydrogel group ($p < 0.05$). At 5 weeks, the bone density of the simvastatin-loaded microsphere hydrogel group was gradually stable and increased with time up to week 8; the bone density of the simvastatin-loaded microsphere hydrogel group was noted to be higher than that of the simvastatin-free hydrogel group ($p < 0.01$). Bone density of the blank MS hydrogel group showed a slight increased trend from the week1 to week 8 after the operation (Fig. 6).

3.5. Histology

Histological observation showed that width and density of newly formed bone tissue in the extraction socket gradually increased with time. Figs. 7 and 8 show histological sections of rat lower incisor extraction socket 1, 2, 5 and 8 weeks after treatment. Many spindle stromal cells and a small number of inflammatory cells were infiltrated in extraction socket 1 week after treatment, and there was no significant difference among the three groups. At 2 weeks, sparse woven bones were found in the extraction socket of the simvastatin-free group, and few osteoblasts were detected around newly formed bone tissue. There was a small amount of new bone formation in the extraction socket of the simvastatin-loaded microsphere gelatin group. In the empty gelatin group, little new bone tissue was formed surrounded by inflammatory cells. At 5 weeks, denser newly formed alveolar bone was detected in the extraction socket in the simvastatin-loaded microsphere gelatin group. In contrast, new bone in the extraction socket in the empty gelatin group was sparse and dispersed, and was restricted to the extraction socket wall with a large number of inflammatory cells. At week 8, significant bone formation was observed in the simvastatin-loaded microsphere gelatin group, and most of extraction socket was occupied by the newly formed bone tissue. Little bone formation was observed in the extraction socket treated with the gelatin hydrogel that was simvastatin-free, and apparent gaps existed among the newly formed bone tissues. The empty gelatin group was surrounded by sparse newly formed bones.

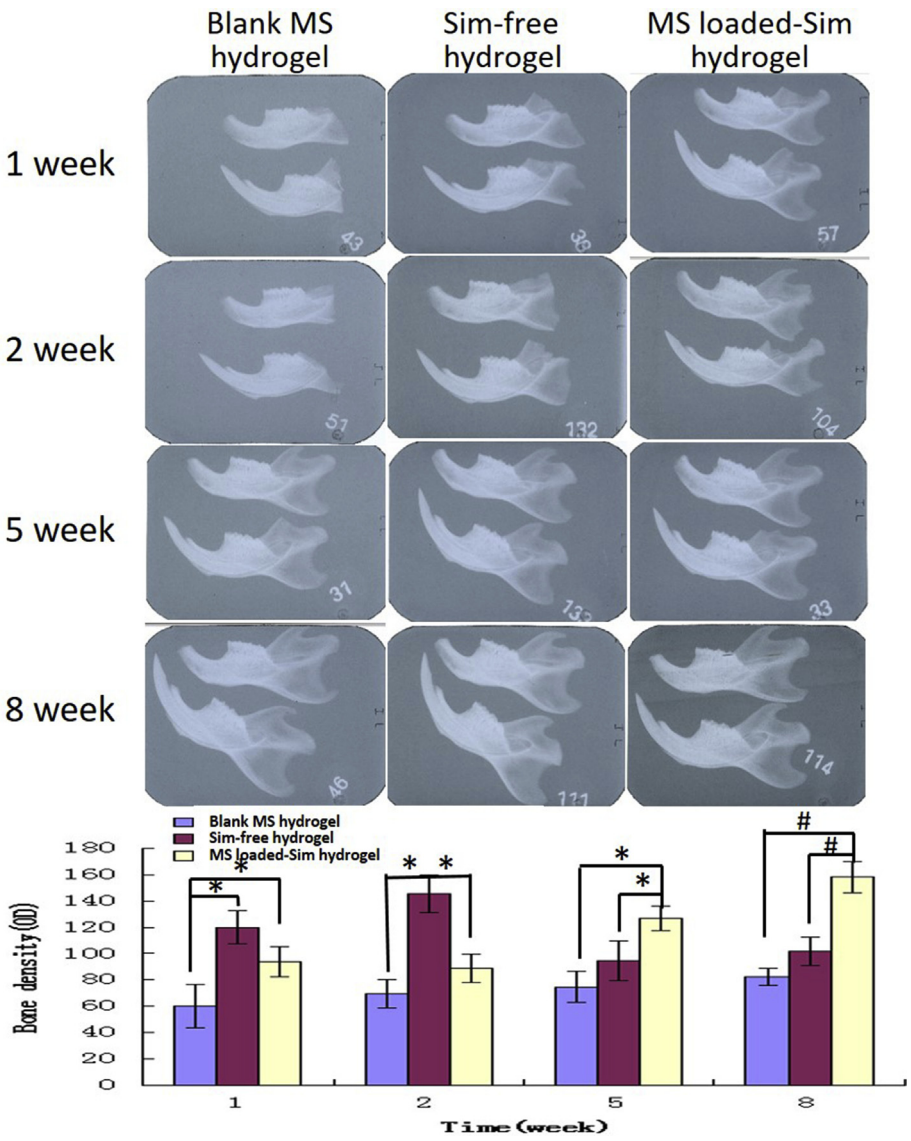


Fig. 6. (A) Soft X-ray photographs of mandibles of blank microspheres (MS) hydrogel, simvastatin (Sim)-free hydrogel, and MS loaded-Sim hydrogel groups. (B) Quantitative analysis of socket bone density. In each image, the upper is the mandible with extracted incisor and the lower is the mandible without extracted incisor (*$p < 0.05$; #$p < 0.01$).

4. Discussion

The resorption of alveolar bone results in many aesthetic and functional problems in edentulous patients. The tooth extraction socket is often used as a bone healing model to estimate new bone formation. Molybdenum-target soft X-rays were used in this study because they are sensitive to small mineralized tissues and can better distinguish a variety of bone trabecular microstructure. In addition, this technique exerts little damage to tissues and allows the use of the same sample for other tests (Kengyelics et al., 2017).

Statins are widely used in clinical practice to lower cholesterol, treat hyperlipemia/hyperlipidemia and arteriosclerosis. Studies in vitro and in vivo have suggested that statin stimulates osteoblasts and promotes bone formation (Mundy et al., 1999; Wu et al., 2008). In this study, we developed an injectable hydrogel system which embedded simvastatin-loaded microspheres, and we tested its effect on bone healing using a rat incisor extraction socket model. The excellent injectability of the hydrogel system facilitated its easy fitting into any irregular extraction socket through a syringe (Fig. 3A). The scaffolding materials in this research are gelatin and PLGA, which are both biodegradable and can be discharged in the form of CO_2 and H_2O. The degradation of the scaffolding materials left space to allow new bone tissue ingrowth. In this work, the scaffolding materials were designed to have a fast degradation rate, which allowed them to be completely degraded in the extraction socket within a few weeks (Fig. 7). Following the bone tissue formation, the bone resorption and remodeling started and was a continuous process (Haggerty et al., 2015).

At the first week, the histological images of the extraction socket in all three groups were of similar bone density, which was probably due to the fact that a blood clot formation in the extraction socket was a necessary step for tooth extraction socket healing (Pang et al., 2015) and bone defect repair (Ma et al., 2018). Changes in alveolar bone mineral density in the blank microspheres hydrogel group were similar with regard to normal extraction socket healing process: the first stage was bone resorption with decreased bone density, followed by gradually enhanced bone formation. In comparison, the bone mineral density in the alveolus increased first, followed by a gradual decrease in the simvastatin-free hydrogel group, which might be related to the fluctuation of

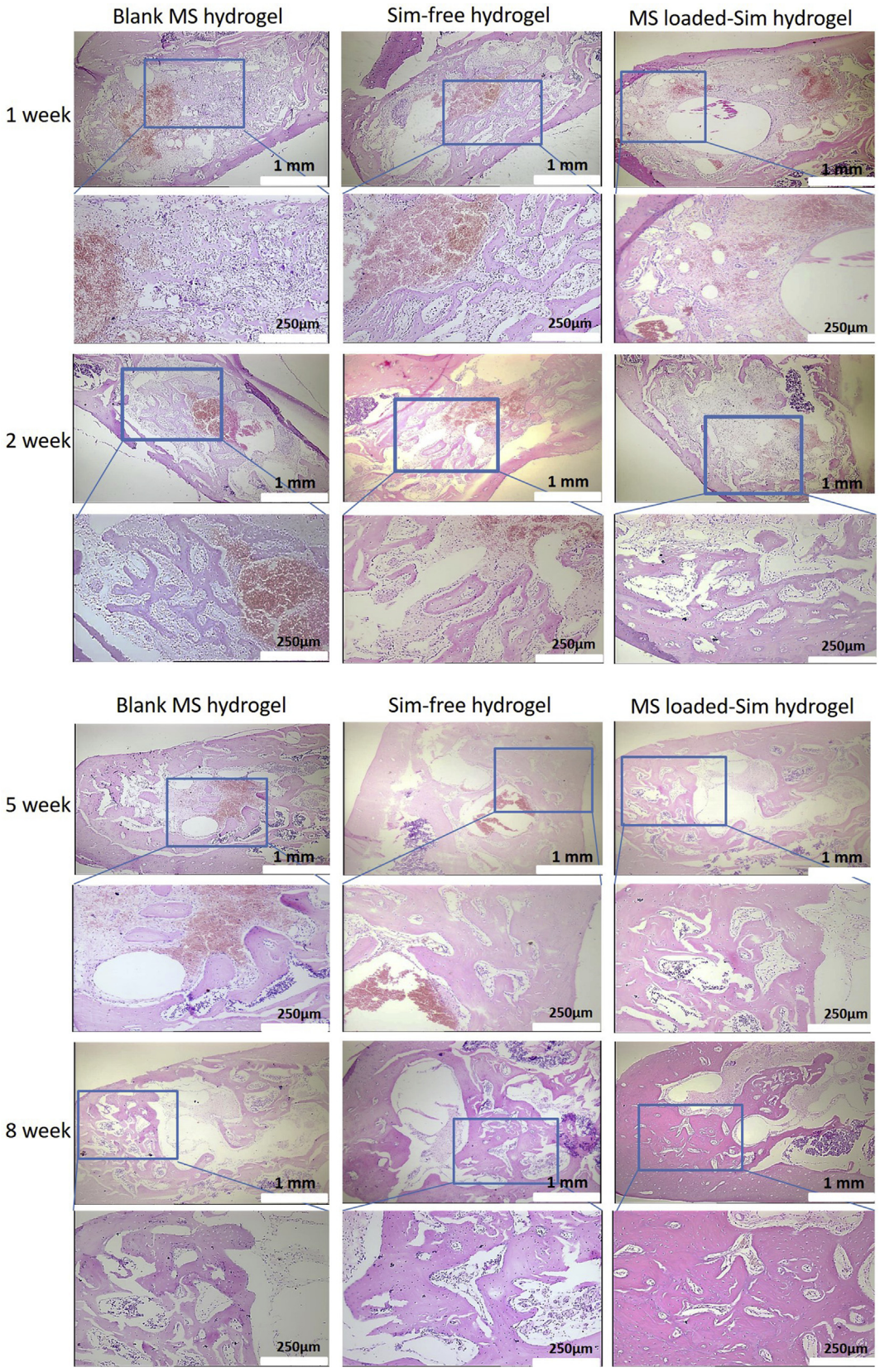


Fig. 7. Hematoxylin and eosin (H&E)–stained mandible sockets of blank microspheres (MS) hydrogel, simvastatin (Sim)-free hydrogel, and MS loaded-Sim hydrogel groups. In the blank MS hydrogel group, newly formed bone volume only slightly increased from week 1 to week 8. In the Sim-free hydrogel group, bone tissue increased at the week 2, then decreased, indicating bone resorption. In contrast, in the MS loaded-Sim hydrogel group, newly formed bone tissue stably increased from week 1 to week 8.

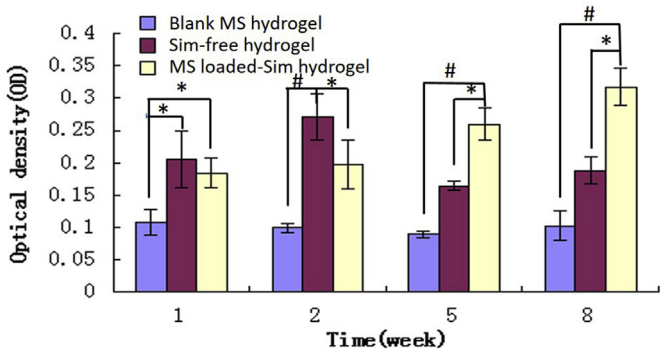


Fig. 8. Quantitative analysis of newly formed bone tissue in the socket of blank microspheres (MS) hydrogel, simvastatin (Sim)-free hydrogel, and MS loaded-Sim hydrogel groups at weeks 1, 2, 5, and 8 (*$p < 0.05$; #$p < 0.01$).

simvastatin in the defect area (Olbricht et al., 1999); the simvastatin from the microsphere hydrogel system was released at a constant rate during the whole period, which led to a continuous osteogenic effect in the simvastatin-loaded microspheres hydrogel group. The bone mineral density of the mandible increased, and the newly formed bone trabecular bone became wider (Fig. 7).

Both bone formation and bone resorption exist in the process of bone defect repair, which was confirmed by the changes in the width and density of new trabecular bone in tooth extraction sockets with the time, drugs and metabolism (El-Rashidy et al., 2017). The histology image shows the inflammatory reaction in blank microspheres hydrogel group was more obvious than other groups that included simvastatin, suggesting that the simvastatin had an anti-inflammatory effect (Gunjiganur Vemanaradhya et al., 2017). Interestingly, in the simvastatin-free hydrogel group, the most intensive bone formation occurred at week 2, followed by the reduction of the bone density until week 8. A possible explanation is that the excessive simvastatin rapidly released from the simvastatin-free hydrogel led to inflammation in the mandible bone (Michael et al., 2002; David Stein et al., 2005), which further induced bone tissue resorption (Hienz et al., 2015; Kheirallah and Almeshaly, 2016). Therefore, it is essential to optimize simvastatin dosages for stimulating maximal bone formation without inducing inflammation.

The preparation process of the injectable simvastatin-loaded microsphere gelatin scaffold is simple. In addition, the scaffold acts as an osteoinductive materials via sustainably releasing simvastatin from the PLGA microspheres that were embedded in the gelatin hydrogel (Pan et al., 2018). Additionally, simvastatin has been reported to have antibacterial properties (Gunjiganur Vemanaradhya et al., 2017). All these factors worked together to lead to a better bone formation using the simvastatin-loaded microsphere gelatin system.

The limitations of the present study include the small numbers of animals in each group and the reporting of only short-term results. To further confirm the effects of simvastatin in the microsphere system on bone regeneration, larger numbers of the animals and long-term time follow-up (eg, 4–6 months) should be included in future studies. In addition, we included only two control groups (a blank microspheres hydrogel group and simvastatin-free hydrogel group) in this study, and the addition of another control group (blank in the tooth extraction socket) would further confirm the effect of the simvastatin released from the microspheres of the simvastatin-loaded hydrogel on bone formation.

5. Conclusion

The present study demonstrated that the gelatin hydrogel containing simvastatin-loaded microspheres led to more bone formation than that of the simvastatin-free and the blank microspheres hydrogel groups after tooth extraction. Therefore, the simvastatin-loaded microsphere system is a promising candidate for bone repair and regeneration.

Conflicts of interest

The authors have stated that they have no conflict of interest.

Acknowledgements

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